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The Influence of Temperature on Streptomycin Inhibition Zones in Agar Cultures

By K. E. COOPER and W. A. GILLESPIE

Bacteriology Laboratories, Bristol University, and Bristol Royal Infirmary

SUMMARY: The diffusion of streptomycin in agar gel seeded with staphylococci as in assay methods, results in a clearly defined zone of inhibition. The time required for this definition to develop is about 2½ hr. at 37°, but varies greatly with temperature and is equal to the lag period plus approximately 4 times the generation time. After 4–5 generations the young colonies become much less susceptible and grow unimpeded by the rapidly increasing concentration of the antibiotic reaching them. Incubation at temperatures below 37°, although without significant effect on the minimum inhibitory concentration, results in larger inhibition zones because of increased generation times.

In assays and sensitivity tests by diffusion methods, errors will occur if organisms under comparison do not multiply at the same rate.

When an antiseptic diffuses into nutrient agar seeded with a sensitive organism (as in the standard diffusion assay methods) there is a race between the drug diffusing into the medium, and the growth of the colonies. At first sight it might seem that the edge of the zone of inhibition occurs at a point where colonies have grown to macroscopically visible size or to full size before the minimum inhibitory concentration of the antiseptic reaches them. This explanation, however, does not account for the sharpness of the edge of the inhibited zone seen with some antibiotics. Experiments with streptomycin indicate that when the young colonies have grown for a certain critical, and quite short, time, they become much less susceptible to the action of streptomycin, and that they continue to grow to full and even to enhanced size in spite of the presence around them of much more than the minimum inhibitory concentration. Thus the edge of the zone is defined before the colonies become visible to the naked eye.

The experiments with streptomycin using the Mayo strain of staphylococcus were concerned in the first place with the effect of temperature on the size of the inhibited zone. The formula which we used was suggested by Cooper & Woodman (1946). This formula relates the distance travelled by the minimum inhibitory concentration of antiseptic in any particular time to the concentration of the antiseptic placed in the cup or on top of the column of agar. It rests on theoretical principles which apply to the tube assay method for streptomycin (described by Mitchison & Spicer, 1949) where the agar seeded with the organism is placed in narrow tubes, and the streptomycin is placed on top of the agar column. After incubation the length of the resulting zone of inhibition is measured with a microscope vernier. In the equation of Cooper & Woodman (1946):

$$x^2 = 4(2{\cdot}30\, DT)\,(\log m_0 - \log m');$$

x = length (mm.) of inhibited zone, m_0 = concentration of antiseptic placed in contact with the agar, m' = minimum inhibiting concentration of the antiseptic in agar, before a critical time (called here T_0) after the start of growth, T = time (hr.) the antiseptic has been diffusing, and D = diffusion coefficient (mm.^{2}/hr.).

In the usual method of assay, antibiotic is added to the tubes *at the moment* when they are warmed to the incubation temperature. Diffusion and growth begin at the same moment, and we propose to call the particular value of T which must be used to fit the theoretical line to the experimental points T_0. Most workers have not determined this constant because, since D is also constant in a particular experiment, DT_0 is the only constant in addition to m' which must be known in order to draw the theoretical curve. This product DT_0 determines the slope of the normal assay line.

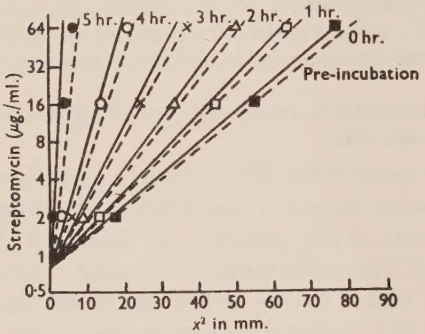

Fig. 1

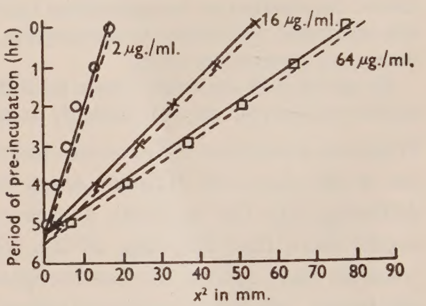

Fig. 2

Fig. 1. Effect of pre-incubation on zones of inhibition of staphylococcus by streptomycin at 27°. Concentration on a logarithmic scale is plotted against x^2, where x = depth of inhibited zone. The hour of pre-incubation represents the length of time before streptomycin was added to the tubes, for each set of determinations. Theoretical lines for $T_0=5{\cdot}5$, – – –, and $T_0=5{\cdot}25$, ——, are given when $D=0{\cdot}84$ mm.^{2}/hr. and $m'=0{\cdot}75\,\mu$g./ml. Period of pre-incubation (hr.): 0, ■; 1, □; 2, △; 3, ×; 4, ○; 5, ●.

Fig. 2. Graph giving the same results as Fig. 1 but showing the constancy of T_0 for different concentrations of streptomycin. $D=0{\cdot}84$ mm.^{2}/hr. $m'=0{\cdot}75\,\mu$g./ml. Theoretical lines $T_0=5{\cdot}5$, – – –, $T_0=5{\cdot}25$, ——. Experimental points, streptomycin μg./ml.: 64, □; 16, ×; 2, ○.

Other assays can be made with a different value of T by pre-incubating the tubes before adding the antibiotic. The experiments of Schmidt & Moyer (1944), as was explained by Cooper & Woodman (1946), were of this type. If incubation and hence growth proceeds for h hr. before the antibiotic is added, then growth will have proceeded for T_0 hr. as soon as T_0-h hr. have elapsed since diffusion started. The assay line which results will be given by using T_0-h, instead of T in the formulae.

A series of such lines is shown in Fig. 1, which records experiments carried out at 27°. When h was increased until it became greater than T_0, no inhibition occurred no matter how much streptomycin was added to the tubes.

These results can be shown in another way by plotting h against x^2 for each concentration m_0 used:

$$h=T_0-\frac{x^2}{9{\cdot}2D\,(\log m_0-\log m')}=T_0-\frac{x^2}{K}.$$

This is shown in Fig. 2 and gives also a straight line for each concentration provided no change in m_0 or m' occurs in the time T_0. When $h = T_0$ then $x^2 = 0$. The method of determining T_0, then, is to find where the x^2/h graph cuts the h axis, and if the theory is correct this point should be *independent of concentration*. It will be seen that almost all the points lie between lines for $T_0 = 5{\cdot}5$ and $T_0 = 5{\cdot}25$ hr.

To obtain this agreement care had to be taken over many technical details, particularly those concerned with temperature control and time. The rapid inoculation of liquid agar at a temperature of 45°, its setting and cooling for a standard time, its placing in the water-bath at the required temperature and the use of antibiotic solution previously warmed to this temperature are examples. Other details are described in the experimental section of the paper.

When corresponding graphs at 37° were plotted the minimum inhibitory concentration m' remained unchanged at 0·75 units, but T_0 was considerably less, namely 2·75 hr. It is indeed remarkable that at this temperature, in as short a time as 3 hr., the colonies of staphylococci became insusceptible to high concentrations of streptomycin, though the individual organisms on subculture appear to be as sensitive as ever.

It is not proposed to show sets of graphs for each temperature investigated, but the effect of temperature on the normal assay curves when $h = 0$ (diffusion and growth starting at the same time), is shown in Fig. 3. Theoretical curves have been drawn from the formula with the concentration $m' = 1$ because, although variations occurred in individual experiments from 0·75 to 1·25, we decided that their importance was doubtful, and that the total results were best expressed by using this mean value. Secondly, we give in Fig. 4 the calculated h/x^2 lines for a concentration of $64\,\mu$g. streptomycin/ml. as this shows the very great effect of temperature on T_0 which increases from 2·25 at 42° to 9·0 at 22°. Both figures show close agreement with the experimental points. The lower temperatures give much larger zones of inhibition.

It will be noticed that in Fig. 4 the lines are not parallel but slightly converging. This convergence is due to the effect of temperature on D, which can be calculated from the effect of temperature upon the viscosity of water. The dependence of D on absolute temperature and viscosity was described in the paper by Cooper & Woodman (1946). The values for the results at 42°, calculated in this way, agree very well with the observed values. Table 1 shows the values of the constants D, T_0 and m' which best fit the whole series of points obtained by experiments conducted at each temperature for different concentrations and at varying periods of pre-incubation. The changes in D and T_0 with temperature are in opposite directions and, though the effect of T_0 is much the greater, the product DT_0 gives a minimum value at 37°. The results at 42° are very similar to those at 37°, though findings at lower temperatures differ greatly. The calculated relative values for D are given in Table 1 and show good agreement.

It was then noticed that the effect of temperature on the generation times of a number of organisms—*Bacterium coli*, streptococci, etc., reported in the literature were proportional approximately to our T_0 values. As no record could be found for the staphylococcus, determinations were made of the generation time of the strain used in the broth base of our agar medium, seeded in the same way with the same number of organisms.

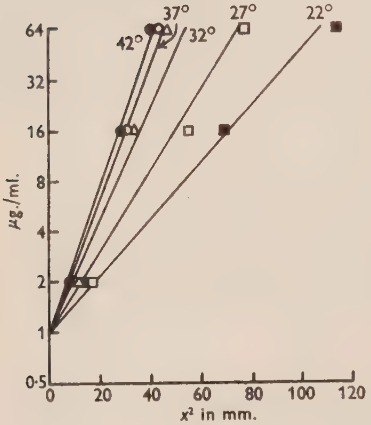

Fig. 3

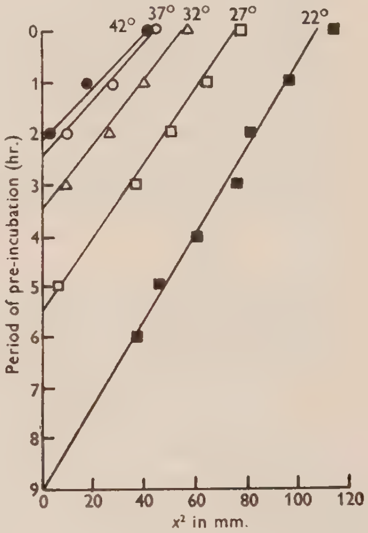

Fig. 4

Fig. 3. Streptomycin inhibition zone with staphylococcus in tubes. The relation of x^2 to log concentration for different temperatures, without pre-incubation. Theoretical lines are drawn with m' independent of temperature and values of D calculated from the values of the viscosity of water for each temperature (see Table 1). $h=0; m'=1$. Experimental points: ●, 42°; ○, 37°; △, 32°; □, 27°; ■, 22°.

Fig. 4. Streptomycin inhibition zones with staphylococcus in tubes, at different temperatures. The influence of temperature on T_0 is shown by the results of pre-incubation with one concentration of streptomycin (64 μg./ml.). The theoretical lines use the calculated relative values of D for the different temperatures and a constant value for the minimum inhibitory concentration $m'=1$. Experimental points: ●, 42°; ○, 37°; △, 32°; □, 27°; ■, 22°.

Table 1. *Influence of temperature on constants of assay curves*

Streptomycin on staphylococcus (Mayo)

Temp. (°)	D observed	D calculated	T_0 observed	DT_0	m'
42	1·15	1·15	2·2	2·53	1·00
37	0·92	1·03	2·5	2·30	0·75
32	0·89	0·92	3·5	3·12	0·90
27	0·84	0·82	5·5	4·63	0·75
22	0·75	0·72	9·0	6·85	1·25

Results in tubes in water-baths at controlled temperature.

Table 2 shows that the ratio of T_0 to the generation time is approximately 5·5 over the range of active growth from 22 to 42°. A still more constant ratio is obtained by allowing for the lag period which was experimentally determined, a correction which reduces it to 4. In other words, the critical change in susceptibility from colonies inhibited by m', to colonies insusceptible to considerably higher concentrations of streptomycin occurs between the development of the 4th and 5th generations of descendants of the original inoculum.

Table 2. *Influence of temperature on generation time and lag period*

Streptomycin on staphylococcus (Mayo)

Temp. (°)	T_0 (hr.)	G.T. (min.)	L. (hr.)	T_0/G.T.	T_0–L./G.T.
42	2·2	25?	0?–0·5?	5·3	5·3–4·1
37	2·5	29	0·8	5·0	3·5
32	3·5	29	1·0	5·4	3·9
27	5·5	54	1·5	6·1	4·4
22	9·0	86	3·0	6·3	4·2
			Average	5·6	4·1

Results in tubes in water-baths at controlled temperature. G.T. = generation time; L. = lag period.

DISCUSSION

The main effect of temperature on the zone of inhibition has been shown to be due to the changed rate of growth of the organism. Effects on minimum inhibitory concentration (m') and on the diffusion coefficient D are of much less importance. T_0 has been found to be given approximately by $T_0 = 4{\cdot}1$ (generation time) + lag period. The value of the minimum inhibitory concentration of streptomycin in agar for the *Staphylococcus aureus* strain Mayo is little affected by temperature. Nevertheless, the size of the inhibited zones of growth is greatly affected because of the large variation in T_0 (and a small variation in D). Between 22 and 42° the formula $x^2 = 4(2{\cdot}30D)\ (T_0 - h)\ (\log m_0 - \log m')$ agrees within the limits of experimental error with the facts investigated, and there seems to be no advantage in using more complicated formulae. The theoretical significance of the resistance of the colony which arises after four generations (with the strength of inoculum used in these experiments) will be discussed elsewhere. It will be of interest to examine other organisms and other antiseptics by these methods. We suspect from the varying nature of the boundary zone between inhibition and growth with different antiseptics, that factors not yet considered may affect the zone. The rate of action of the antiseptic at concentrations near m' has not been considered in the simplified theory proposed, which assumed that concentrations of m' and above inhibited instantly growth up to time T_0, but were without effect after time T_0. It has also been assumed that concentrations below m' are without any effect. These are obviously only partially correct assumptions, and it is not surprising therefore that zones obtained with other organisms and antibiotics may not always be as easy to measure, or agree so well with the theory as results obtained with streptomycin and staphylococcus.

Apart from the theoretical implications of these facts, there are some practical conclusions to be drawn from these experiments. One is that when antibiotic assays are done by the agar-diffusion methods, care must be taken to ensure that the temperature of the medium is exactly the same in all the tubes and plates used. For this reason, tubes are in general better than plates, and a water-bath than an incubator. There is also a practical bearing on sensitivity tests done by the agar-diffusion method (as in a ditch or cup plate). If two organisms which are being compared on the same plate have significantly different generation times, conclusions about sensitivity may be erroneous. Such apparent differences in sensitivity might arise if the medium used were relatively more suitable for one of the organisms.

EXPERIMENTAL DETAILS

Determination of inhibition zones. The assay method of Mitchison & Spicer (1949) was used, with some modifications.

Medium. Equal parts of nutrient (infusion) agar and a 1 % solution of peptone (Evans), adjusted to pH 7·8 and stored in 50 ml. quantities.

Test organism. The 'Mayo' strain of *Staph. aureus.*

Agar diffusion tubes. Narrow tubes (3 mm. internal diameter) as used for assays.

Method of incubation. Accurately controlled water-baths which could be adjusted between 22 and 42° were used.

Procedure. The object of the experiments was to obtain a set of streptomycin standard assay curves at each temperature, by varying the length of time for which growth was allowed to occur before the streptomycin solutions were placed in contact with the columns of seeded agar. Thus there were obtained, at each temperature, curves for 0, 1, 2, 3, etc., hr. of pre-incubation. The temperatures used were 22, 27, 32, 37 and 42°. Two such sets of curves were obtained in each experiment, one at 37° and one at another of the temperatures. The 37° results thus served as controls for the comparisons of curves obtained on different occasions. Particular attention was given to the exact timing of periods of pre-incubation, accurate temperature control and uniform treatment of the test organism.

Three standard solutions of streptomycin in distilled water were used in constructing the curves, their concentrations being 2, 16 and 64μg. streptomycin base/ml. These were made up at the beginning of each experiment from a stock 10,000μg./ml. solution kept at 4°, and were warmed in the water-bath before adding to the assay tubes. For each standard solution, three assay tubes were used, so that to obtain a single curve a row of nine tubes was required. One such row was put up for each period of pre-incubation.

To set up an experiment, one bottle of medium was melted and its contents distributed in 9·5 ml. amounts in 1 oz. screw-capped McCartney bottles which were placed in a 45° water-bath. Sufficient time was allowed for temperature equalization. Two-tenths ml. of an overnight (37°) infusion broth culture of the staphylococcus was added to 10 ml. sterile distilled water at 37°, mixed and kept in the incubator. Five-tenths ml. of the diluted culture was added to 1 bottle of melted medium, which was thoroughly mixed, replaced in the 45° water-bath and used to fill the eighteen sterile assay tubes comprising two rows (one to be incubated at 37° and one at the other temperature). After filling, the tubes were not re-plugged. The times of commencing and completing the filling of each row were noted. The tubes were fixed by means of plasticine vertically on the bench (at 18 to 22°) for 15 min. (During this period another bottle of medium was seeded and filled into tubes.) The tubes were then placed in Wassermann racks in the appropriate water-baths. The warmed streptomycin solutions were added quickly, either at once or after the planned periods of pre-incubation, by means of fine Pasteur pipettes and without removing the assay tubes completely from the bath. After incubation overnight, the zones of inhibition were measured by means of a microscope vernier scale

Determination of generation time. The medium was the same as that used for the determination of inhibition zones, except that infusion broth replaced the nutrient agar. An inoculum of the same size was used and the seeded medium held first at 45° and then on the bench for the same periods as when determining inhibition zones before incubating in the water-bath at the required temperature. Viable counts (Miles & Misra, 1938) were done at hourly intervals. The results were plotted and the lag period and generation time were determined from the intersection and slope of the best straight line with the horizontal through the level of the initial count.

We should like to express our thanks to Mrs N. Webster for technical assistance.

REFERENCES

Cooper, K. E. & Woodman, D. (1946). The diffusion of antiseptics through agar gels, with special reference to the agar cup assay method of estimating the activity of penicillin. *J. Path. Bact.* 58, 75.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of blood. *J. Hyg., Camb.* 38, 732.

Mitchison, D. A. & Spicer, C. C. (1949). A method of estimating streptomycin in serum and other body fluids by diffusion through agar enclosed in glass tubes. *J. gen. Microbiol.* 3, 184.

Schmidt, W. H. & Moyer, A. J. (1944). Penicillin. I. Methods of assay. *J. Bact.* 47, 199.

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Cooper, K. E. & Linton, A. H. (1952). *J. gen. Microbiol.* 7, 8–17

The Importance of the Temperature during the Early Hours of Incubation of Agar Plates in Assays

By K. E. COOPER and A. H. LINTON
Bacteriology Laboratories, Bristol University

With a note by K. E. Cooper on the theory of inhibition zones

SUMMARY: The time taken for agar plates to reach incubator temperature under different conditions was determined by thermocouple measurements. From 1 to 5 hr. was taken to reach within 1° of the incubator temperature, according to their mass, position, stacking and original temperature. Delay in heating slowed the growth of organisms and produced larger inhibition zones due to diffusing antiseptics. This leads to errors in sensitivity and thus in assay work unless technique is standardized to overcome these differences. Deviations from the calculations resulting from the formula of Cooper & Woodman (1946) can be overcome by such standardization.

An appendix discusses the theory of inhibition zones and compares the formulae proposed by Cooper & Woodman (1946), by Vesterdal (1947) and by Mitchison & Spicer (1949).

The present investigation was commenced because of some anomalous results, which did not fit the expected straight lines (see Fig. 1), obtained by the use of the agar-cup method. We had applied the formula of Cooper & Woodman (1946) to assay results with 8 mm. cups, though it was strictly applicable on theoretical grounds to either tubes or cups of infinite diameter—i.e. to gutters in plates. Vesterdal (1947) proposed a different formula for cups which theoretically was true only for infinitely small cups—i.e. point sources of diffusion, though he used 5 mm. cups in his experimental work. Vesterdal did not test his results by the method we have used, namely by finding the effect of pre-incubation on the size of the zone of inhibition.

Cooper & Gillespie (1952) showed in tube experiments on streptomycin that the Cooper & Woodman formula gives a straight line when h, the period of pre-incubation in hours, is plotted against x^2 (x = width of zone of inhibition). Vesterdal's formula, as it applies to small cups, takes into account the exhaustion of the antiseptic which results from its diffusion out of the cup. The fall in concentration with time causes the h/x^2 graph to be curved instead of straight. On the other hand, log concentration/x^2 is still a straight line because the edge of the ring is formed at a single time (which we have called T_0).

Experiments with plates

Fig. 1 shows the experimental results obtained with surface cultures on plates, made in trying to compare these two formulae. Much to our surprise, the experimental lines were much more curved than expected from either theory. This was not due to more rapid exhaustion of the penicillin by absorption into the agar, as in tube experiments with similar amounts of antiseptics we obtained the expected straight lines. The method we were using of removing plates from the incubator to add the antiseptic obviously caused a temporary cooling of the cultures, and to see whether this was the cause we investigated the effect of temperature on our results. Cooper & Gillespie (1952) showed with streptomycin that a lowering of temperature had a marked effect on the constant T_0, and a slight effect upon the diffusion coefficient D. The product DT therefore increases as the temperature falls, and the size of the zone of inhibition is thus increased for a particular concentration.

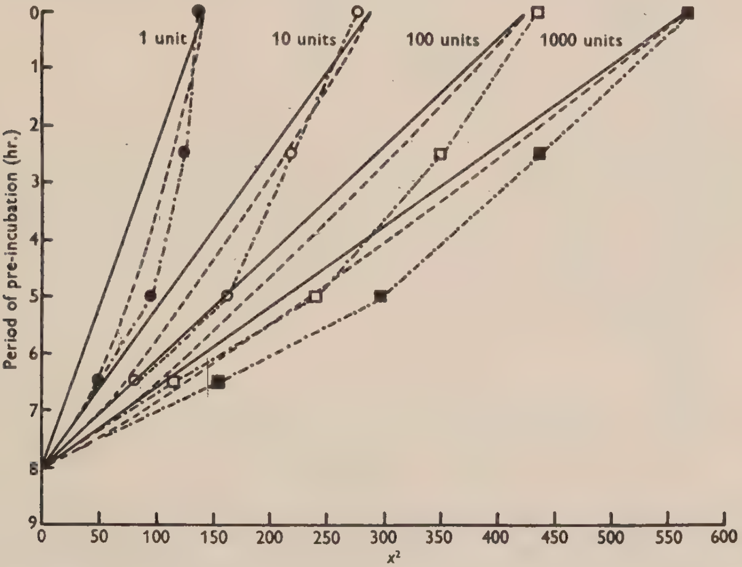

Fig. 1. The effect of pre-incubation on inhibition zones due to the action of penicillin on staphylococcus. Theoretical lines according to the formula of Cooper & Woodman, ——, and according to Vesterdal's formula, – – –, are compared with experimental points, ·–·–·, obtained by a technique described in the text which gives erroneous results. Concentration of penicillin: 1 u., ●; 10 u., ○; 100 u., □ ; 1000 u., ■.

The events depicted in Fig. 1 were as follows. When $h=0$ the plates were not taken out of the incubator after the penicillin was added, and we should expect that the T_0 value will approximate to a definite value below that for 37° depending on the rapidity with which the plates warm up. When $h=2$ hr. the plates were removed (after 2 hr. in the incubator) and inevitably cooled while the antiseptic was added. When $h=4$ hr. the plates, necessarily more adequately heated (if more than 2 hr. be required to reach 37°) will have suffered greater cooling and the average temperature will be lower than when $h=0$, or $h=2$ and DT_0 much greater.

When $h=8$ hr. the plates were similarly cooled, but the growth preventing a zone of inhibition was already completed and the temperature changes up to this moment were the same as for $h=0$. Thus we suspected from the results shown in Fig. 1 that plates in the incubator took longer than 2 hr. to reach approximately 37° and that the curvature of these lines was due to the differing rates at which the temperature was attained.

In order to investigate the actual temperature attained by growing colonies in agar plates, a thermocouple was constructed for us by Dr Burge of the Physics Department, in which four junctions of copper and constantan were held by a mica frame with Perspex bolts, a few mm. above the bottom of a Petri dish. Agar was poured over this and allowed to set.

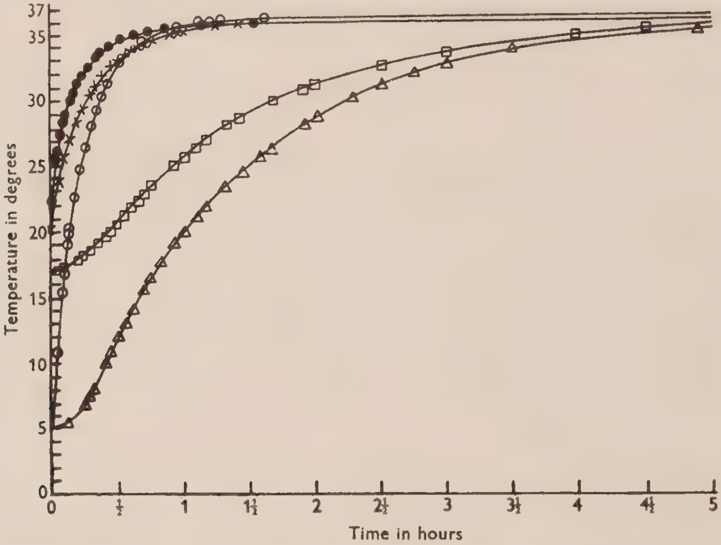

Fig. 2. The temperature of agar plates after placing in the incubator at 37°. ●—●, agar 7 mm. thick in plate on shelf, warmed from room temp. 20°; ○—○, agar 7 mm. thick in plate on shelf, warmed from refrigerator temp.; ×—×, agar 14 mm. thick in plate on shelf, warmed from room temp. 20°; □—□, agar 14 mm. thick in plate in middle of a pile of 5 plates from 17°; △—△, agar 14 mm. thick in plate in middle of piles of plates from 5°.

Fig. 2 shows the actual temperatures in the agar plotted against time. Two of the curves show the results for a single agar plate (*a*) heated from room temperature, and (*b*) from refrigerator temperature. Rapid heating occurred and the temperature attained in 1 hr. was less than a degree below the final equilibrium temperature in both cases. A third curve with a single plate shows the slight amount of slowing of the process which occurred when the mass of the plate (i.e. the amount to heat up) was increased by using thicker agar. But when the plate was included in a pile of plates, the close contact with plates above and below made the pile act as a single unit of much increased mass and the rate of heating was very considerably decreased. Even in a single pile of five plates, the middle plate (the most slowly heated) required 4 hr. to get to within 1° of the final temperature. When the pile is surrounded by others 5 hr. may be required.

Theory of temperature control

The curves in Fig. 2 agree during the main part of their course with what would be expected from Newton's law of cooling. For a body suspended in air the amount of heat lost is stated to be proportional to the time and the temperature difference between the body and the air (if the temperature difference is small). If the specific heat of the body does not change with temperature, then not only the heat loss, but the actual fall in temperature, will be proportional to the temperature difference and time:

$$\frac{d\theta}{dt} = -r\theta,$$

$$\frac{d\theta}{\theta} = -r\,dt,$$

$$\ln\theta = rt + \ln\theta_0,$$

$$\log_{10}\theta - \log_{10}\theta_0 = -rt(0{\cdot}4343),$$

where θ = temperature difference, t = time, r = cooling rate/min./degree difference, θ_0 = temperature difference when $t=0$. Plotting the logarithm of the temperature difference between the plate and the incubator against time should then give a straight line if Newton's law is followed. Fig. 3 shows how good the agreement is, deviations only occurring (*a*) in the first few minutes until a steady state is reached or (*b*) when temperature differences become less than one degree and the experimental errors are exaggerated by the logarithmic scale.

The mechanism by which the plate is heated is mainly by convection of the air, by some conduction through the shelf or plates below or above the experimental plate and, to a small extent only, by radiation. The conduction mechanism seems the most adequate when the plate is directly on a metal shelf. Insulating material or another plate placed beneath the experimental plate slowed down the process of heat exchange. Protection of the plate from convection currents by placing another on top, made another considerable decrease in rate of heating. It follows that in a pile of five plates the process is quickest with the bottom plate and slowest with the middle plate. The experimental values of the constant r are given in Table 1.

Table 1. *Effect of plate arrangements in incubator on rate of heating*

Values of r (cooling rate/min./degree difference of temperature)

		Plate* heated to 37°				
		From room temp. 20°		**From refrig. temp. 0–4°**		**After cooling† 5 min. Agar 7 mm.**
		No agar	**Agar 7 mm.**	**Agar 14 mm.**	**Agar 7 mm.**	
Single plates:	**On shelf**	0·0756	0·0475	0·0460	0·0595	—
	On box	—	0·0388	—	0·0506	0·0361
Pile of 5 plates:	**Bottom**	—	—	0·0303	—	—
	Top	—	—	0·0163	—	—
	Middle	0·0146	—	0·0129	—	—
5 piles × 5 plates	**Middle**	—	—	0·0107	0·0134	—

* Glass Petri dish approx. 10 cm. external diameter.

† After cooling 5 min. refers to plates previously heated to 37° and then placed at room temperature (20°) for this period.

The results obtained were approximately what would be expected from theory, but it was noticed that the value of the constant r was somewhat greater when the initial temperature difference was great, e.g. when plates were heated from refrigerator temperature, and somewhat smaller when heated from temperatures higher than room temperature, e.g. for a plate only cooled for 5 min. from 37°. Many factors such as alterations of conductivity and specific heat of the materials concerned may complicate the absolute value of the constant r. Radiant heat exchange follows a fourth power law, and may be playing some part when the temperature difference is greatest.

The importance of temperature control in diffusion methods

It will readily be seen from these results and the effects of lower temperature demonstrated in Cooper & Gillespie's (1952) paper, that exactly reproducible results at constant temperatures will be difficult to obtain by the plate method. The use of thin tubes to contain the assay medium (Mitchison & Spicer, 1949) allows of much better temperature control, especially when incubated in a water-bath. Similar control with plates can probably only be accomplished by working in an incubator room, preferably at 27° instead of 37°, as the larger values of DT at that temperature will give larger rings.

That our findings did explain the curvature found in Fig. 1 we finally showed by making T_0 determinations with a concentration of 10u. penicillin/ml. in each cup with plates at constant temperature. This was done by means of experiments made with all the plates arranged singly on the shelf of the incubator, and in which the penicillin was added in the incubator. The opening of the door produced only a slight temporary cooling of the plates, particularly at the front of the shelf.

We also made experiments by removing all plates from the incubator every 2 hr., and adding penicillin to those that required it. All plates were thus subjected as nearly as possible to the same temperature changes. Seeded agar plates were used to minimize the effect of surface cooling on lifting the cover. The resulting points fell accurately in a straight line, and showed no tendency to even the curvature expected from Vesterdal's formula (see Fig. 4). The 8 mm. cups therefore contain enough penicillin for the concentration gradients to be unaffected by loss from the cup in the first 8–11 hr. during which the ring is formed. (This may be no longer true for concentrations of penicillin lower than 10u./ml. as it was noticed that the rings with 8 hr. pre-incubation had become very ill defined with the size of inoculum used.)

The T_0 values for 10u. penicillin/ml. in these different experiments are given in Table 2.

An unexpected result was the greater value found for bottom plates in a pile compared to top plates, but other factors such as carrying the piled plates through the air to the bench, and the warmth of the hands holding the plates may have disturbed this result.

A value is given for surface colonies on the single plates, because the double zoning resulting from the larger ring at the surface compared to the inhibition in the depth, was definitely marked in these experiments. It may be that the admission of cold air to the surface of the plates caused greater chilling than in the depths and hence a greater value of T_0. We have constantly observed much greater zones of inhibition with surface inoculated plates than with poured plates or tubes.

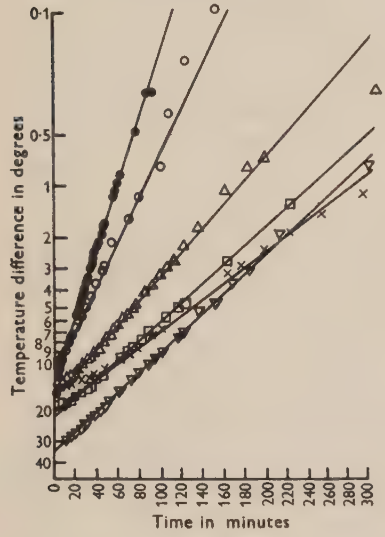

Fig. 3

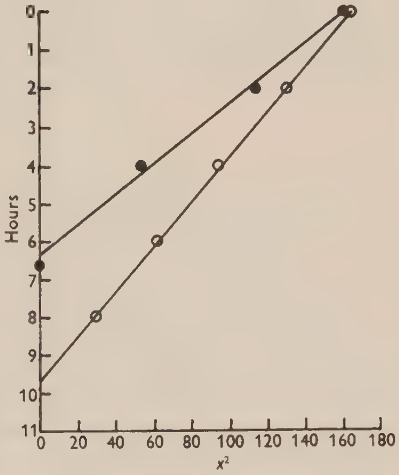

Fig. 4

Fig. 3. The difference of temperature (logarithmic scale) between the incubator and an agar plate. ●, single plate on shelf; ○, bottom plate of a pile of five plates; △, top plate of a pile of five plates; □, middle plate of a pile of five plates; ×, middle plate of a number of piles of plates, from 20°; ▽, middle plate of a number of piles of plates, from 5°.

Fig. 4. The influence of treatment of the plate on T_0 value, obtained by the action of penicillin on staphylococcus. ●, single plates not removed from the incubator; ○, average value for piles of plates removed every 2 hr.

Table 2. *Influence of treatment of plate on T_0 value*

Poured plates	T_0 (hr.)	Poured plates	T_0 (hr.)
Piles of 5 plates removed every 2 hr.:		Single plates not removed:	
Middle plates	10·7	Deep colonies, average	6·4
Top plates	9·1	Deep colonies, front	6·4
Bottom plates	9·4	Deep colonies, back	6·0
Average	9·7	Superficial colonies	8·0

NOTE ON THE THEORY OF INHIBITION ZONES

By K. E. Cooper

In the first paper of this series Cooper & Woodman (1946) showed that crystal violet diffused into an agar column in such a way that a particular concentration m' would be achieved at time T, at a point distance x from the surface, when a concentration m_0 was placed at the surface at time 0, where

$$x^2 = 4DT\,2{\cdot}30(\log m_0 - \log m'). \tag{1a}$$

D is the diffusion coefficient of the crystal violet in the water interspaces of the agar gel, and varies with the temperature and viscosity of the solute.

It was next shown that the edge of the zone of inhibition, formed when the agar contained a growing culture of organisms, was determined by a particular value of concentration of antiseptic in agar (m') and a particular value of T, which depended on how long the organism had grown since inoculation. When incubation of the culture was started simultaneously with the beginning of diffusion of the antiseptic we have called this value T_0. If the culture was pre-incubated for h hr., then the value of T (the time the antiseptic has diffused) becomes T_0-h. The edge of the zone of inhibition is therefore given by

$$x^2=4D(T_0-h)\,2{\cdot}30(\log m_0-\log m'). \tag{1b}$$

This formula was shown to apply not only to crystal violet and penicillin but to streptomycin (Cooper & Gillespie, 1952). In the case of penicillin, diffusion took place from the circular cup of a plate assay, instead of into tubes, although it was pointed out that this was an approximation only accurate for cups of large diameter (or to gutters). Nevertheless, cups of 5–8 mm. diameter seemed sufficient, within the limits of experimental error, to allow of application of the formula. The concentration of antiseptic in the cup (m_0) must be reasonably constant for some hours. It was also pointed out that disturbances from osmosis, evaporation and liquid flow might be expected to make inaccuracies when the zones of inhibition were very small, i.e. when m_0 approaches m' or h approaches T_0.

Vesterdal (1947) considered the problem of the *small* cup of area A in which the penicillin is continually diminishing in concentration as it escapes by diffusion. Using the same symbols as above and converting his formula to the logarithmic form, it becomes

$$x^2=4DT2{\cdot}30\left(\log m_0-\log C_p-\log\frac{4\pi DT}{A}\right), \tag{2}$$

where C_p is the smallest bacteriostatic concentration of the antiseptic.

It will be seen that when $m'=\frac{4\pi DT}{A}C_p$ this formula becomes identical with (1). This, however, is only so when T is constant, or when A is large. When x^2 is plotted against $\log m_0$, then m' is the point where the straight-line graph (on either formula) cuts the $\log m_0$ axis. On formula (1) this point is constant and independent of T. On formula (2) a different point will be obtained for each value of T. That is if T is varied, by allowing the antiseptic to diffuse before incubation of the culture is started or by incubating the culture before the antiseptic is added ($\pm h$), the resulting series of straight lines obtained, one for each value of T, will meet at one point on the $\log m_0$ axis for formula (1) and not with formula (2).

If a graph of x^2 against h is constructed for each concentration m_0 in the cup, (1) gives a straight line, but (2) gives a curve. It was hoped that the variation of x^2 with h would thus give an important means of elucidating the limitation of the application of the two formulae. Moreover, only by performing experiments with different hours of pre-incubation or pre-diffusion can the value of

T_0 be determined. However, the experimental errors involved due to the lack of temperature control made comparison difficult. Cooper & Linton's results suggest that 8 mm. cups give straight lines at constant temperature. There seems therefore little necessity to use the more complicated and cumbersome formula of Vesterdal, for normal assay techniques. His explanation of the marked zones of partial growth at the edge of the ring, for low concentrations of antiseptic, as due to the exhaustion of the supply in the cup, before the ring is formed, is a valuable theoretical contribution.

Mitchison & Spicer (1949) expanded the fundamental diffusion formula as a series and proposed a third formula which, if we use the same symbols as above, is:

$$x^2 = 4DT\,2\cdot3\left(\log m_0 - \log(2C\sqrt{\pi}) - \log\frac{x}{2\sqrt{(DT)}}\right), \tag{3}$$

where $\frac{x}{2\sqrt{(DT)}}$ must be fairly large. On the other hand, when x is large $\log\frac{x}{2\sqrt{(DT)}}$ becomes negligible compared to $\frac{x^2}{4DT}$, and this formula then becomes the same as (1) if $m' = 2C\sqrt{\pi}$. Moreover, though they show that there is a definite deviation from the straight line $x^2/\log m_0$ for low concentrations (below 2 μg./ml.) of streptomycin, they agree that it is doubtful if any proposed formulae agree for small values of x. Cooper & Woodman (1946) pointed out other disturbing factors for low concentrations of antiseptic in their original paper. For concentrations of streptomycin 4–256 μg./ml. formula (1) holds. Since the method of assay was in tubes, formula (2) is not applicable except by putting $a = \infty$ when it reduces to the formula of Cooper & Woodman. It thus seems to us that for assays by the tube or gutter plate method formula (1) is the simplest, and that the further elaborations suggested by other workers will not give any more accurate approximations to the experimental results. The normal assay method is concerned with only one value of T, which is determined by the conditions of the assay, i.e. $T = T_0$. Usually not even T_0 is determined, because for any one set of experimental conditions operating on one batch of media on one day DT_0 will be a constant, and the formula:

$$x^2 = 4DT_0\,2\cdot30(\log m_0 - \log m')$$

contains only the measurements x and the concentrations m_0 as variables. The measurement of x therefore for two concentrations m_0 determines the graph and enables the point m' where the straight line cuts the axis and the slope of the line ($4DT_0\,2\cdot30$) to be evaluated.

The importance of determining T_0 is illustrated by the relationship discovered between its value and the rate of growth of the organism at different temperatures, by Cooper & Gillespie (1952). With the strength of inoculum used in these experiments it was shown T_0 = lag period + 4·1 (generation time). over a temperature range of 22–44° for *Staphylococcus aureus* strain Mayo. Factors in addition to temperature which might affect either the lag period (previous treatment of the inoculum) or the generation time (media composition) were kept as constant as possible. It appears that under these conditions, after some four generations of growth, the bacteria are able to deal adequately with concentrations of antiseptic greater than m', so that growth can continue at least for some time and produce large colonies. It is obvious that there must be limits to this remarkable change in resistance which occurs at time T_0, for high concentrations of antiseptics are obviously capable of eventually killing growths on solid media. Inhibition of growth after time T_0 may be the explanation of the diminished sharpness of the edge of the zone, in tubes, that occurs with high concentrations of crystal violet for example. The assay of penicillin in tubes is a much less satisfactory process than that of streptomycin, and other time/concentration factors than are considered in this simple formula may be influential.

No adequate explanation of the increase in resistance that the bacterial growth on solid media shows, after T_0 hr., has yet been given. Although as a result of the geometrical progression of bacterial growth, the exponential increase in concentration of antiseptic results eventually in a decreased amount of antiseptic per organism in parts of the agar where the minimum concentration m' does not quickly reach the growth, this is not sufficient explanation. It must be remembered that the pre-incubation experiments, in which h was varied from 0 to T_0, also gave rise to resistant growth after 4 generations. This resistance is still evident, and zones of inhibition are not formed when $h = T_0$, even when very high concentrations of antiseptic are placed in contact with the agar. Subcultures of such growth show no greater resistance to the antiseptic and we are not evidently dealing with either mutation or adaptation. Colonies of the size of 20–60 organisms seem too small to suggest inadequate penetration of the antiseptic to the individual organisms. Experiments to investigate the possible production of antagonistic substances, either present in the media or the organism have not been helpful so far, as the results are complicated by the effect of these substances on the generation time. A slowing down of growth would increase the size of the zones, and neutralize the possible effect of antagonistic substances reducing the rings.

Dubos (1945) quoted numerous examples of change in resistance of organisms at the stage of 'physiological youth'. The effect of cold shock, of heat, and of the action of antiseptics is correlated with the maximal metabolic activity, cell volume and reproduction rate in the early hours of growth. It seems reasonable to suggest that T_0 is a fairly accurate determination of the end of physiological youth, and that more attention should be given to the profound change that takes place in a growing culture at this time.

Experiments with inocula in exhausted culture media, or in previously uninoculated broth showed no significant influence on T_0. Dilution of the inoculum has a marked effect on T_0, but little on m'. The effect of dilution of the staphylococcus on the action of streptomycin is well illustrated by the published graph of Mitchison & Spicer (1949). In this case, there is a rough proportionality between the slopes of the lines (proportional to T_0) and the $\sqrt[3]{N}$, where N is the concentration of organisms in the inoculum. If the edge of the zone of inhibition is judged by a standard opacity then we might expect the readings to be proportional to the linear density of the colonies, as in this case (the size of the colonies at T_0 being constant). This would only be true if the nearness of the colonies to each other were without effect on the generation time in the first few hours. Such an effect will thus probably be limited to special cases and light inocula. The results of Schmidt & Moyer (1944) on the effect of inoculum size on penicillin plate assays, and our own results suggest that often the effect is less marked than even the $\sqrt[3]{N}$.

Assuming m' and D constant in Mitchison & Spicer's results, the slopes of their graph give the following results:

Dilution	1/4000	1/2000	1/1000	1/500	1/250
Relative N	1	2	4	8	16
$\sqrt[3]{N}$	1	1·26	1·59	2·0	2·52
Slope S	1·478	1·191	1·080	0·746	0·525
$S\sqrt[3]{N}$	1·478	1·490	1·715	1·492	1·340
Ratio to average 1·505	0·98	0·99	1·14	0·99	0·89
Error	−0·02	−0·01	+0·14	−0·01	−0·11

The formula used in this series of papers explains in a qualitative way other factors influencing the size of the zone of inhibition. Schmidt & Moyer (1944) showed that refrigeration of the inoculated plates increased the zone of inhibition. The stopping of growth with the probable increase of lag period, coupled with the increased time required by refrigerated plates to reach 37° on incubation would increase the value of T_0 and the size of the rings.

The effect of nutritional factors remains to be investigated, but any factor influencing lag or generation time will affect the result.

REFERENCES

COOPER, K. E. & GILLESPIE, W. (1952). The influence of temperature on streptomycin inhibition zones in agar cultures. *J. gen. Microbiol.* 7, 1.

COOPER, K. E. & WOODMAN, D. (1946). The diffusion of antiseptics through agar gels, with special reference to the agar cup assay method of estimating the activity of penicillin. *J. Path. Bact.* 58, 75.

DUBOS, R. J. (1945). *The Bacterial Cell*, pp. 137–43. Harvard University Press.

MITCHISON, D. A. & SPICER, C. G. (1949). A method of estimating streptomycin in serum and other body fluids by diffusion through agar enclosed in glass tubes. *J. gen. Microbiol.* 3, 184.

SCHMIDT, W. H. & MOYER, A. J. (1944). Penicillin. I. Method of assay. *J. Bact.* 47, 199.

VESTERDAL, J. (1947). Studies on the inhibition zones observed in the agar cup method for penicillin assay. *Acta path. microbiol. scand.* 24, 272.

(*Received* 26 *September* 1951)

Wade, H. E. (1952). *J. gen. Microbiol.* 7, 18–23

Observations on the Growth Phases of *Escherichia coli*, American Type 'B'

By H. E. WADE

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SUMMARY: Observations on the growth phases of *Escherichia coli* (*Bacterium coli*), American type 'B', on aerated media are reported. On casein-casein-yeast (CCY) medium the culture passes through the following phases: (*a*) an initial stationary phase; (*b*) a logarithmic lag phase; (*c*) a phase of rapid cell division; (*d*) a phase of equal division and growth rates; (*e*) a phase of diminishing growth and division rates. The average cell weight increases regularly through phases (*a*) and (*b*) to a maximum. It falls during phase (*c*) to a lower value at phase (*d*) and decreases finally to its initial value when the growth rate decreases more slowly than the division rate during phase (*e*). Similar phases can be recognized during growth on 2 % peptone. The onset of the phase of rapid cell division appears to be stimulated by the concentration of cell material.

It has long been recognized that the growth and multiplication of bacteria can be divided into phases. Those recognized by most workers are the seven proposed by Buchanan (1918), but several anomalies remained until Clarke & Ruehl (1919) and Henrici (1923) demonstrated the marked changes in cell volume that take place in the course of cultivation, and Hershey (1938, 1939) gave a clear distinction between growth, i.e. increase in weight of protoplasm and multiplication, i.e. increase in number of viable units. While investigating fluctuations in the chemical composition of bacteria during their *early growth* it was necessary to have these growth phases clearly established. The present paper sets out some observations made in an attempt to do this.

METHODS

A strain of *Escherichia coli* (*Bacterium coli*), American type 'B', was used and its growth characteristics were followed during cultivation in aerated CCY medium (Gladstone & Fildes, 1940) at 37°. Seed cultures were grown in 1 l. cylindrical all-glass vessels in which adequate aeration was provided through a sintered glass element at the base. The experimental cultures were grown in 10 l. aspirators and aerated by means of a P 35 'Aerox' element. Air was passed through both cultures at the rate of 2 l./min./l. medium. Silicone DC Antifoam 'A' (Midland Silicones Ltd.) was used to diminish foaming.

Where practicable the increase in weight of cell material was estimated by direct determination of cell dry weight—otherwise it was estimated indirectly by light absorption. For direct determination volumes of culture sufficient to give 15–30 mg. dry cells were treated with formaldehyde (final concentration 1 % w/v), centrifuged, washed with 0·9 % w/v sodium chloride containing 1 % w/v formaldehyde and then with 0·05 % sodium chloride and dried at 105° in small tared tubes. The direct determinations were carried out in triplicate. For indirect determination by light absorption, since no influence of cell size on the relationship between turbidity and dry weight could be detected, the light absorption of samples was measured by a Hilger Spekker absorptiometer and the values converted into dry weight cells/unit vol. by means of a calibration curve.

Cell division was followed by making total counts by means of a Petroff-Hausser counting chamber. The average dry weight/cell was calculated from the dry weights and total count/unit volume. The minimum cell dry weight varied between $3{\cdot}5\times10^{-10}$ and 5×10^{-10} mg./cell.

The experiments were conducted in the following way: 10 ml. of CCY medium were inoculated with a loopful of a Robertson's broth culture and incubated overnight. This culture served as inoculum for the seed culture of 700 ml. CCY medium which was grown in the vessel described above. When no further increase in cell material was observed, the required weight of cells was centrifuged down, washed once in saline and used to inoculate the experimental medium, the growth characteristics of which were to be determined. To avoid loss in viability through storage of the washed seed the seed culture was set up in an electrically heated vaccine bath at room temperature and heat applied by means of a time switch during the night. This ensured that by the following morning the seed culture had just approached the maximum stationary phase and that the seed prepared from it could be used immediately to inoculate the experimental culture. The average 95 % confidence interval for cell dry weight determination was ±2 %; that for the total cell counts was ±5 to 8 %.

RESULTS

Fig. 1 shows the variation of total cell weight, division rate and average cell weight of a culture inoculated to an initial cell concentration of *c.* $2{\cdot}5\times10^{8}$ cells/ml. and illustrates the first divergence from the conventional growth phases. The initial stationary phase is not followed by a phase of accelerating division (lag phase of Buchanan, 1918) but by a phase of suboptimal logarithmic increase ('logarithmic lag' phase) which results in the average cell dry weight increasing to about three times the initial value at *c.* 110 min. Subsequent decrease in the rate of formation of cell material relative to the division rate results in a decrease of the average cell weight. Pl. 1 compares cells taken from this culture at 400 min., when no further increase in concentration of cell material could be detected (Pl. 1, fig. 1), with cells from the end of the 'logarithmic lag' phase at 110 min. (Pl. 1, fig. 2).

Fig. 2 sets out the results obtained from an experiment when the inoculum was *c.* 5×10^{7} cells/ml. The 'logarithmic lag' phase was followed by a phase of rapid cell division which exceeded the growth rate and decreased the average cell weight to about twice the minimum. There followed a phase in which division and growth rate were equal, in consequence of which average cell weight remained constant.

Another culture was inoculated to a concentration of *c.* 10^{6} cells/ml. Fig. 3 shows the changes that took place after the concentration of cell material had reached the lower limit of the absorptiometer, when the degree of turbidity could be measured. At 210 min. the average cell weight was *c.* 4 times the minimum, and the culture was in the phase of rapid cell division; the phase of equal growth and division rates that followed began at about the same concentration of cell material as in the experiment illustrated in Fig. 2.

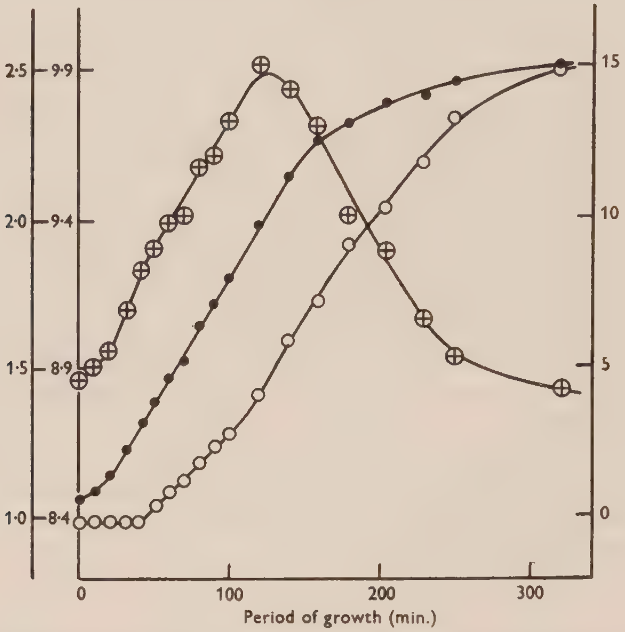

Fig. 1. Growth of *Esch. coli* on aerated CCY at 37°; inoculum *c.* $2 \cdot 5 \times 10^8$ cells/ml. Ordinates: outer left, log mg. dry weight cells/100 ml. medium, —●—●—; inner, left, log total number cells/ml., —○—○—; right, average cell weight (mg. $\times 10^{-10}$), —⊕—⊕—.

These phases, and the changes in growth rate (G.R.), division rate (D.R.) and average cell weight (C.W.) that characterize them (illustrated in Fig. 2) can be summarized as follows:

(*a*) *Stationary phase.* G.R. maximal and logarithmic after a short lag; D.R. zero; C.W. increasing.

(*b*) *'Logarithmic lag' phase.* G.R. maximum and logarithmic; D.R. logarithmic, slower than G.R.; C.W. as (*a*).

(*c*) *Phase of rapid cell division.* D.R. as (*b*); D.R. greater than G.R.; C.W. decreasing.

(*d*) *Phase of equal* G.R. *and* D.R. G.R. as (*b*); D.R. equal to G.R.; C.W. constant.

(*e*) *Phase of negative growth acceleration.* G.R. decreasing; D.R. decreasing more slowly than G.R.; C.W. decreasing to minimum.

Similar phases were recognized during cultivation in 2 % peptone medium. On a defined medium with ammonium lactate base (Cohen & Anderson, 1946) an inflexion to a faster rate of growth at *c.* 100 min. was frequently observed. On tryptic meat broth the initial maximum rate of increase in dry weight was of short duration and the growth rate slowly declined, due probably to depletion of metabolites necessary for optimum growth. This was suggested by changes in the amount and kind of the ninhydrin-positive components (separated on paper chromatograms) of the medium which were observed during cultivation.

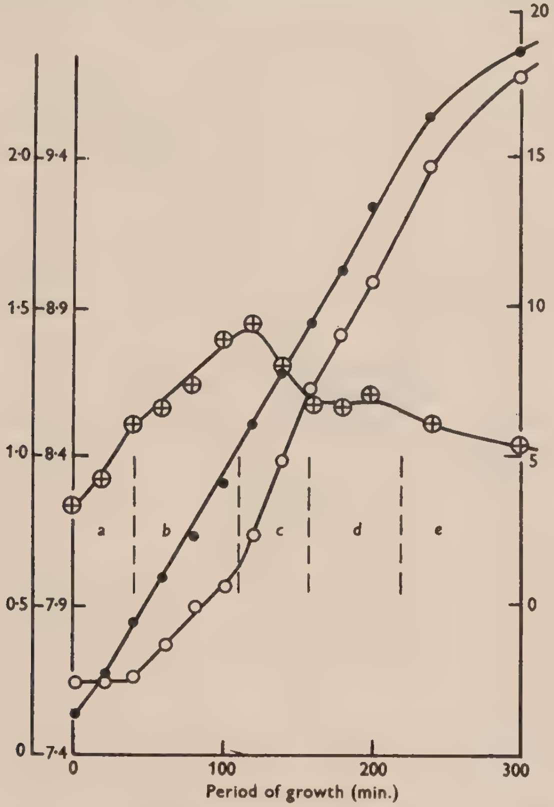

Fig. 2. Growth of *Esch. coli* on aerated CCY at 37°; inoculum *c.* 5×10^7 cells/ml. Ordinates: outer left, log mg. dry weight cells/100 ml. medium, —●—●—; inner left, log total number cells/ml., —○—○—; right, average cell weight (mg. $\times 10^{-10}$), —⊕—⊕—. Letters refer to growth phases described on p. 20.

In spite of the irregularities that occur on the defined medium and on tryptic meat broth the existence in these cultures of phases similar to those described above was suggested by the changes that occurred in the average cell weight which decreased from the maximum in two stages corresponding with phases (*c*) and (*e*) observed in a CCY culture. A further characteristic common to the cultures examined was that the onset of the phase of equal growth and division rates, when present, occurred at *c.* 30 mg. dry weight/100 ml. medium.

DISCUSSION

The appearance of a 'logarithmic lag' phase was unexpected, and no report of a similar observation could be found in the literature. Examination of the cells towards the end of this phase revealed the presence of few large cells, several times the length of the majority (Pl. 1, fig. 2). By assuming that a proportion of the initial population is capable of a decreased regular division rate but of normal regular growth, i.e. increase in total cell mass, the formation of these large cells could account for the presence of this phase. It is likely that the rapid cell division observed is due to division of these large cells, since, first, the average cell weight decreases to the value expected if all the cells had behaved alike, and, secondly, growth and division rates are equal in the next phase, suggesting that cells are no longer increasing in weight without dividing.

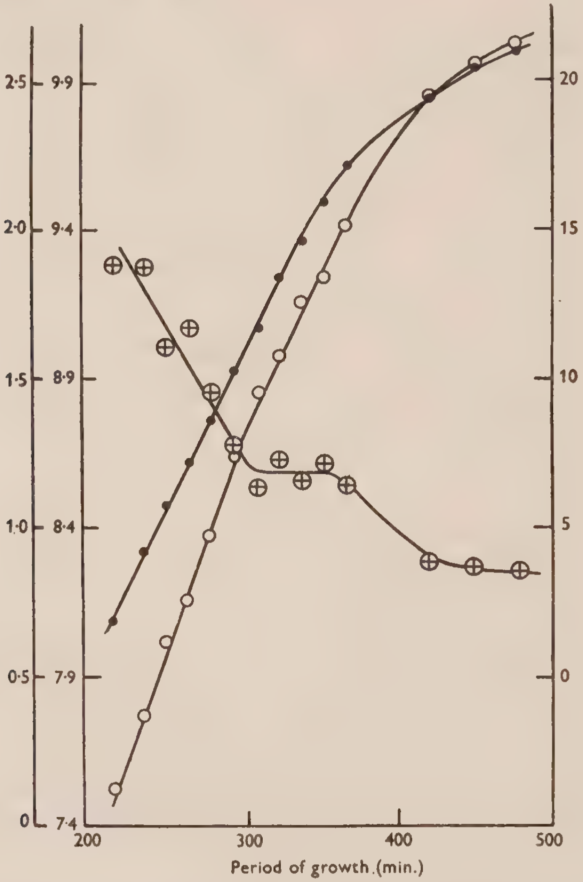

Fig. 3. Growth of *Esch. coli* on aerated CCY at 37°; inoculum *c.* 10^6 cells/ml. Ordinates: outer left, log mg. dry weight cells/100 ml. medium, —●—●—; inner left, log total number cells/ml., —○—○—; right, average cell weight (mg. × 10^{-10}), —⊕—⊕—.

Other workers have reported division rates in excess of growth rates, but the application of the above explanation to their results is questionable. The significance of this phase, however, is naturally bound up with the factors responsible for the diminution in cell volume. It is of interest to recall to what influence other workers have attributed cell volume reduction. Bayne-Jones & Adolph (1932), who followed growth, division and volume of *Esch. coli* by

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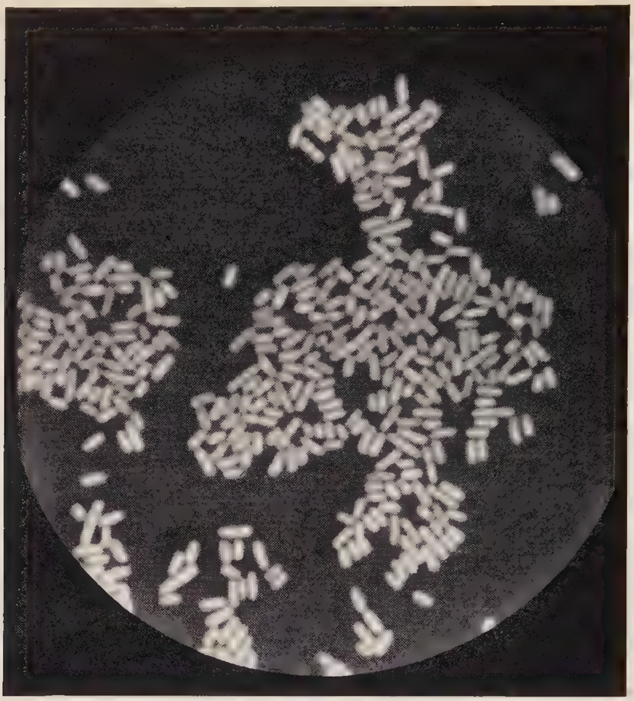

Fig. 1

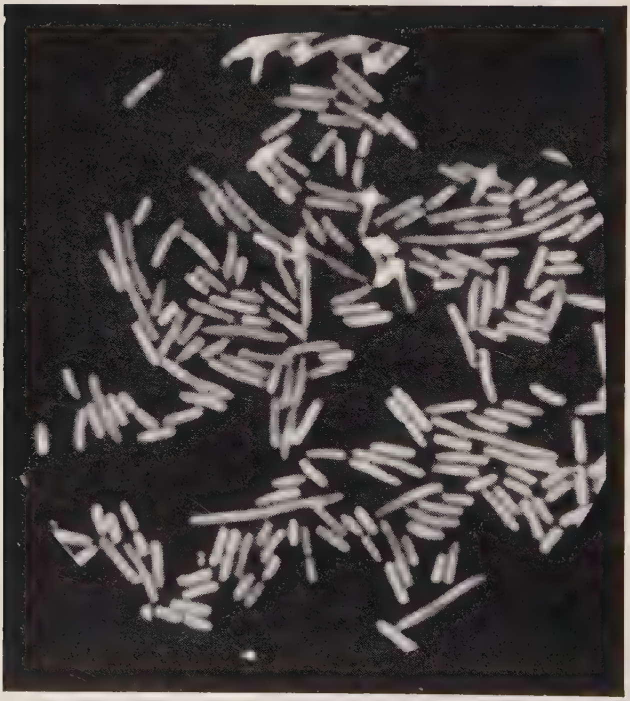

Fig. 2

H. E. WADE—GROWTH PHASES OF *ESCH. COLI*. PLATE 1 motion pictures, observed a correlation between decrease in growth rate and decrease in cell volume. Huntington & Winslow (1936) came to the conclusion that cell volume decreased at the 'log phase' (the phase of rapid cell division). How far the growth rate affected this decrease is uncertain, as viable cell numbers alone were followed. Hershey (1938), who grew *Esch. coli* in static culture in beef extract broth and estimated cell numbers by plate counts, observed a decrease of cell nitrogen due to a decline in growth rate and to a rapid cell division rate. The experiments reported here suggest that decrease of cell weight can be effected independently by both these factors.

Fig. 3 shows that when the number of generations is increased by decreasing the size of the inoculum the onset of the phase of equal division and growth rates occurs at about the same concentration of cell material and that expansion occurs in earlier phases. This suggests that towards the end of growth the increasing scarcity of metabolites is felt more keenly by the large cells through their smaller surface area/volume ratio and prompts them to divide (forming the phase of rapid cell division) to the size of the majority at the phase of equal growth and division rates.

I am pleased to acknowledge the assistance of Sgt. D. M. Morgan. I wish to thank Dr D. W. Henderson for his encouragement and advice, and Dr D. Herbert, Major L. H. Kent and Mr E. O. Powell for helpful criticisms of this paper. The statistical treatment of results was carried out under the guidance of Mr S. Peto. The microphotographs were taken by Miss Nora K. Harris.

REFERENCES

Bayne-Jones, S. & Adolph, E. F. (1932). Growth in size of micro-organisms measured from motion pictures. III. *Bacterium coli. J. cell. comp. Physiol.* **2**, 329.

Buchanan, R. E. (1918). Life phases in a bacterial culture. *J. infect. Dis.* **23**, 109.

Clarke, P. F. & Ruehl, W. M. (1919). Morphological changes during the growth of bacteria. *J. Bact.* **4**, 615.

Cohen, S. S. & Anderson, T. F. (1946). Chemical studies on host-virus interactions. I. The effect of bacteriophage adsorption on the multiplication of its host, *Esch. coli B. J. exp. Med.* **84**, 511.

Gladstone, G. P. & Fildes, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

Henrici, A. T. (1923). A statistical study of the form and growth of *Bacterium coli. Proc. Soc. exp. Biol., N.Y.* **21**, 215.

Hershey, A. D. (1938). Factors limiting bacterial growth. II. Growth without lag in *Bacterium coli* cultures. *Proc. Soc. exp. Biol., N.Y.* **38**, 127.

Hershey, A. D. (1939). Factors limiting bacterial growth. IV. The age of the parent culture and the rate of growth of transplants of *Escherichia coli. J. Bact.* **37**, 285.

Huntington, E. & Winslow, C. E. A. (1936). Cell size and metabolic activity.at various phases of the bacterial culture cycle. *J. Bact.* **33**, 123.

EXPLANATION OF PLATE

Fig. 1. Comparison of *Esch. coli* growing on CCY at 37° at the end of growth (*c.* 400 min.).

Fig. 2. Comparison of *Esch. coli* growing on CCY at 37° towards the end of the 'logarithmic lag' phase (*c.* 110 min.).

(Photographed on nigrosin relief, ×2000.)

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Wade, H. E. (1952). *J. gen. Microbiol.* 7, 24–30

Variation in the Phosphorus Content of *Escherichia coli* during Cultivation

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SUMMARY: Wide fluctuations of phosphorus concentration in the cells occur during the cultivation of *Escherichia coli* (*Bacterium coli*) on aerated media. The concentration of cell phosphorus increases rapidly during the stationary phase and early lag phase to a peak which coincides with the formation of half the first generation; it declines until the end of this generation and increases once again to a second peak, declining subsequently when growth and division rates fall off. Analyses of the cells during cultivation show that the different concentrations of ribonucleic acid largely account for these variations. The association of the initial phosphorus peak with the first generation has been observed during the cultivation of *Esch. coli* on several liquid media and on CCY medium at different temperatures. The increase in phosphorus concentration during the first generation can be associated quantitatively with the increase in average cell weight.

Variations in the phosphorus concentration of bacteria at different periods of growth were recorded by Leach (1906) and by Dawson (1919), but the bacteria used were for the most part derived from agar surfaces and were in consequence of ill-defined metabolic states. Later investigators used liquid cultures but usually confined their investigations to measurements of specific phosphate esters. The present investigation of changes in phosphorus concentration in cells during growth was intended to give not only an indication of gross changes that take place in the concentration of various phosphorus-containing constituents but also an indication of where more detailed analyses might most profitably be carried out.

METHODS

The organism used in these experiments was a strain of *Escherichia coli* (*Bacterium coli*), American type 'B'. The methods of cultivation used are given in Wade (1952). Cells used for inoculating experimental cultures were derived from a culture grown in the same medium and at the same temperature as the experimental culture, and harvested when the maximum concentration of cell material/ml. medium was just reached. These cells were washed once before use. This procedure almost abolished the growth lag, i.e. lag in the increase in cell mass, in all the cultures to be described.

Samples from experimental cultures were delivered into a sufficient volume of 36 % (w/v) formaldehyde to give a final concentration of 1 % (w/v) HCHO. In addition to inhibiting further growth the formaldehyde retarded an apparent decrease in phosphorus concentration during subsequent washings, a decrease which was more severe in cells from young cultures than in those from mature cultures. Volumes of these samples sufficient to provide 15–30 mg. dry weight of cells were centrifuged down, taken up into formol saline, transferred to tared

8 ml. Pyrex test-tubes (7–8 g.) and made up to 5 ml. with washings. The cells were centrifuged down in an angle centrifuge and washed finally in 5 ml. 0·05 % saline. The tubes were dried for 15–17 hr. at 100–105°, cooled and weighed. The determinations were carried out in triplicate and gave a mean coefficient of variation of ± 0·49 %, i.e. 95 % confidence interval of ± 2 %.

Incinerations for phosphorus determination were carried out in the same tubes, using sulphuric and nitric acids. Estimations were carried out according to the method of Fiske & SubbaRow (1925), and are expressed as percentage cell dry weight. The mean coefficient of variation of these determinations was ± 0·54 %, i.e. 95 % confidence interval of ± 2·3 %.

Loss of phosphate from the formaldehyde-treated cells in the time required to wash them prior to analysis was *c.* 1·5 % of the total phosphorus. The loss was similar in actively dividing cells and in 'resting' cells. In view of the small quantity involved no correction was made for this initial loss in the analyses described below.

Separation into various phosphate fractions was carried out by the methods of Schneider (1945). The cells were extracted with trichloroacetic acid and ethanol/ether mixture to remove acid-soluble phosphates and fat-soluble phosphate; nucleic acids were estimated by sugar estimations against yeast ribonucleic acid (RNA) and thymus deoxyribonucleic acid (DNA) standards. RNA was estimated by its colour formation with aniline (Tracey, 1950) and DNA by its colour formation with diphenylamine (Morse & Carter, 1949). Estimation of the nucleic acids by their sugar reactions was used in preference to a phosphorus fractionation technique because of the small weight of material available from samples. In order to facilitate a direct comparison of the changes which occurred in individual phosphorus fractions it was desirable to express the nucleic acids in terms of phosphorus/cell dry weight in common with other phosphate fractions. To effect the conversion the following procedure was adopted. Cells from a mature culture were analysed by the Schmidt & Thannhauser (1945) technique to determine the phosphorus associated with each nucleic acid; a determination was also made by the Schneider (1945) technique to determine the equivalent of yeast RNA and thymus DNA present. The ratios of these two determinations enabled results to be expressed as phosphorus concentration of cell material and made the determinations independent of nucleic acid standards. The closeness of the values of total phosphorus concentration of the dry cell material, as calculated by addition of individual phosphorus fractions to the directly determined values, justified conversion in this manner (Fig. 4).

Total cell counts were made with a Petroff-Hausser counter; the 95 % confidence interval for these results was ± 6–8 %. Cell weight was calculated from dry-weight determinations and total cell counts.

RESULTS

Growth and % total phosphorus/cell dry wt. (% TP/dry wt.) were followed during culture in CCY medium (Gladstone & Fildes, 1940), tryptic meat broth (TMB), and ammonium lactate medium at 37° inoculated to an initial cell concentration of 5×10^8–10^9 cells/ml. During growth on CCY (Fig. 1) the % TP/dry wt. increased and remained high until the growth rate decreased. Similar but smaller variations were observed on ammonium lactate medium (Fig. 2), but on this medium the % TP/dry wt. curve was complicated by an unexpected decrease, repeatedly obtained, at *c.* 100 min.

Fig. 3 shows the variation of TP/dry wt. in two CCY cultures inoculated with *c.* 2×10^8 cells/ml. The phosphorus curve shows a marked decrease in % TP/dry wt., with the first maximum at the time of first division.

Fractionations into various phosphate fractions were carried out on samples taken from the culture described in Fig. 3, Exp. (*a*); the results of these fractionations are shown in Fig. 4.

On TMB and 2 % peptone (two other media that supported rapid growth) decreases in size of inocula to *c.* 2×10^8 cells/ml. produced similar results. The initial % TP/dry wt. peak found during growth on ammonium lactate could also be associated with the first generation, but the magnitude of the increase was much smaller.

There appeared at first sight to be a correlation between growth rate and the increase in % TP/dry wt. at the first generation. Fig. 5, which illustrates the results of several experiments using media that supported growth rates of 0·05–2·7 doubling cell dry wt./hr., indicated a direct relationship.

The growth rate was now varied on CCY by varying the temperature; Fig. 6 shows the changes in % TP/dry wt. during growth at 23°. An initial peak associated with the first generation was observed, but the increase in % TP/dry wt. remained the same as that observed on a CCY culture at 37°. Further experiments showed that on CCY the increase in % TP/dry wt. remained the same over a range of temperatures 21–39·5° representing growth rates of 0·05–2·0 doubling cell dry wt./hr. (Fig. 7).

That % TP/dry wt. was more closely related to cell weight was suggested by the differences in duration of stationary phases observed in the cultures described (Figs. 5 and 7). A plot of the increase in average cell weight against the increase of % TP/dry wt. when half the first generation had been formed showed a direct relationship between the two and that the results illustrated in Figs. 5 and 7 could be accommodated on the one graph (Fig. 8).

DISCUSSION

There is considerable evidence in the literature that the RNA content of living cells is different at different times during growth; that the total phosphorus concentration should be affected is not therefore surprising. The results of phosphorus fractionations carried out on *Esch. coli* cells during growth (Fig. 4) have shown that changes in RNA content are in fact almost entirely responsible for the extensive changes in phosphorus concentration. The results described above may be compared with those of workers who have determined RNA throughout by the sugar reaction or who have determined the fluctuation of nucleotides in bacteria by ultra-violet absorption.

Caldwell, Mackor & Hinshelwood (1950) obtained a correlation between RNA concentration in *Bact. lactis aerogenes* and the growth rate, when the

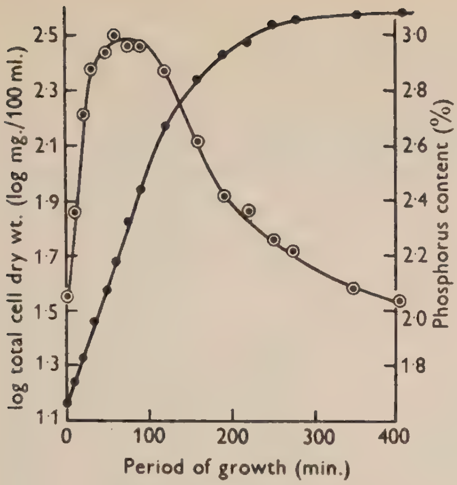

Fig. 1

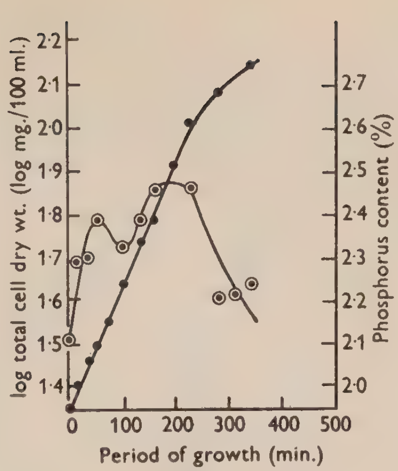

Fig. 2

Fig. 1. Variation of phosphorus content during growth on CCY at 37°. Log mg. dry wt. cells/100 ml. medium, —●—●—; total phosphorus % cell dry wt., —⊙—⊙—.

Fig. 2. Variation of phosphorus content during growth on ammonium lactate medium at 37°. Log mg. dry wt. cells/100 ml. medium, —●—●—; total phosphorus % cell dry wt., —⊙—⊙—.

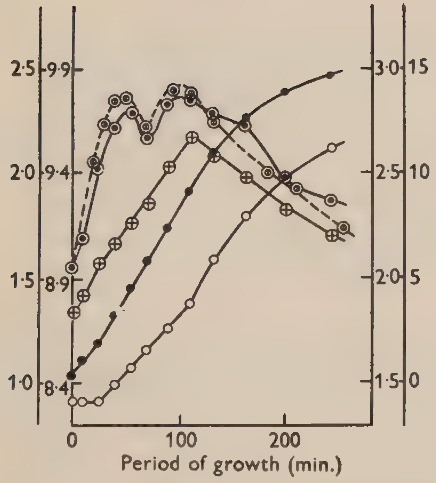

Fig. 3

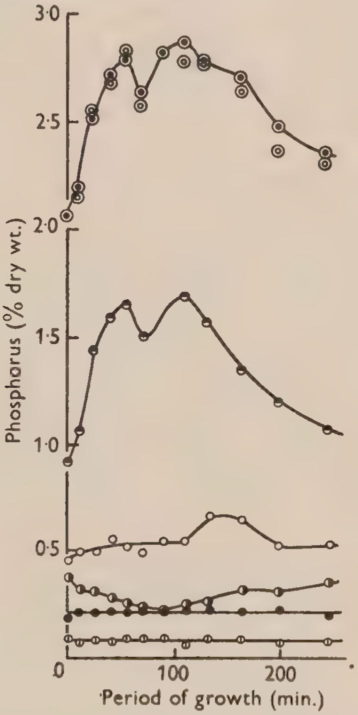

Fig. 4

Fig. 3. Variation of phosphorus content during growth on CCY at 37°; inoculum *c.* 2×10^8 cells/ml. *Exp.* (*a*) Ordinates: outer left, log mg. dry wt. cells/100 ml. medium, —●—●—; inner left, log total cells/ml., —○—○—; outer right, average cell wt. (mg. $\times 10^{-10}$), —⊕—⊕—; inner right, total phosphorus % cell dry wt., —⊙—⊙—. *Exp.* (*b*): total phosphorus % cell dry wt., —◎—◎—.

Fig. 4. Variation of phosphorus fractions during growth on CCY at 37°. Protein P, —⦶—⦶—; fat soluble P, —●—●—; acid soluble P, —○—○—; DNA phosphorus, —◑—◑—; RNA phosphorus, —◓—◓—; calculated total P, —◎—◎—; determined total P, —⊙—⊙—.

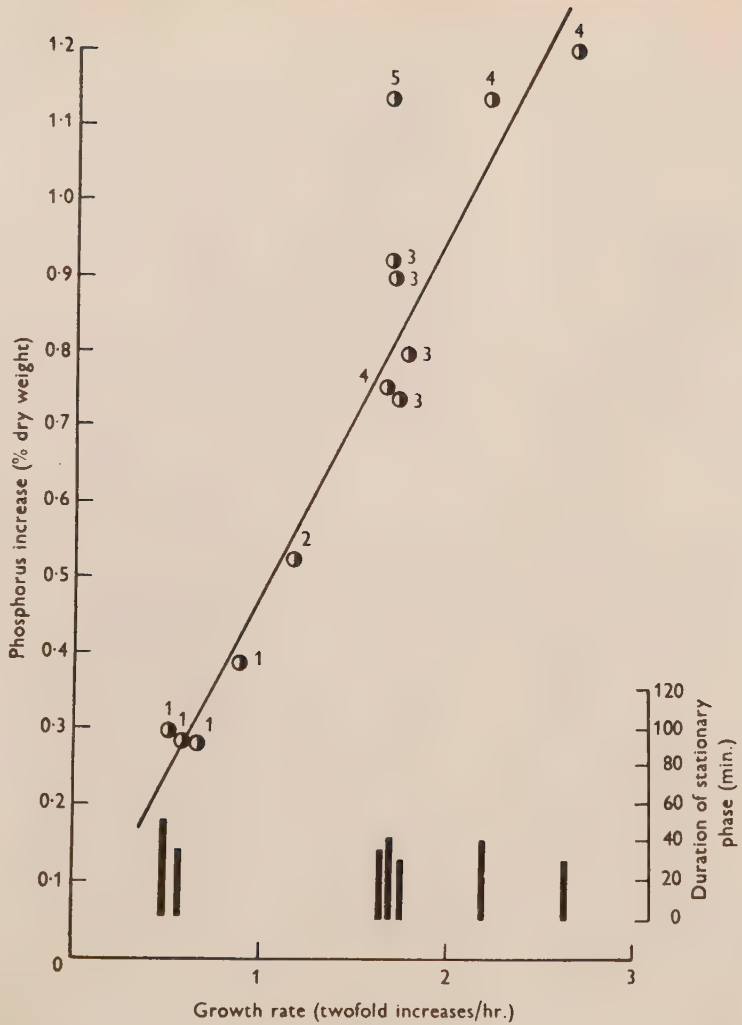

Fig. 5

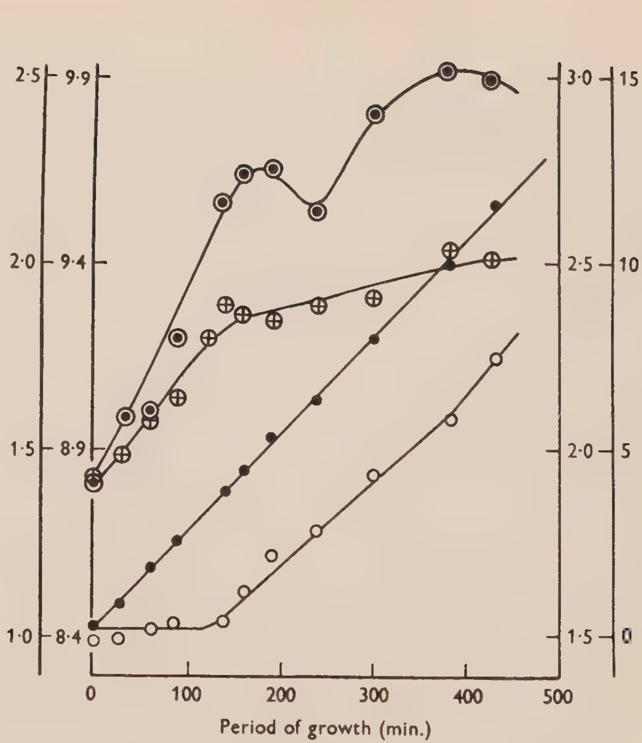

Fig. 6

Fig. 5. Comparison of growth rates and increase in total phosphorus content when half the first generation is formed. The growth rate was varied by employing different media at 37°. Additional data refer to duration of the stationary phases at the respective growth rates. Media: (1) ammonium lactate medium; (2) TMB + 0·5 % glucose; (3) CCY medium; (4) TMB; (5) 2 % peptone medium. A regression line was computed and is given.

Fig. 6. Variation of phosphorus content during growth on CCY at 23°; inoculum *c.* 2·5 × 10^8 cells/ml. Ordinates: outer left, log mg. dry wt. cells/100 ml. medium, —●—●—; inner left, log total cells/ml., —○—○—; outer right, average cell wt. (mg. × 10^{-10}), —⊕—⊕—; inner right, total phosphorus % cell dry wt., —⊙—⊙—.

latter was varied by alterations to the medium. Fig. 5 representing this relationship for cells of the first generation confirms this for *Esch. coli*. However, the results of individual experiments have shown that at the time of inoculation the growth rate becomes maximum almost immediately, whereas

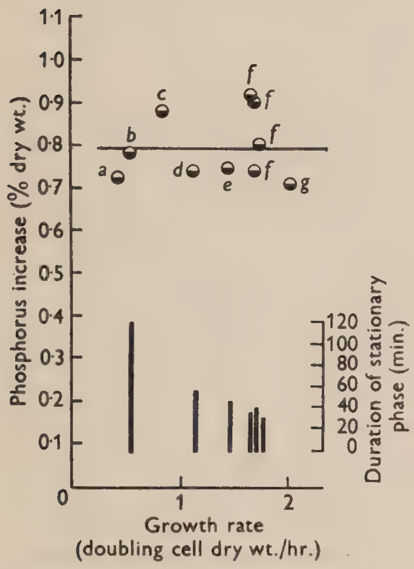

Fig. 7

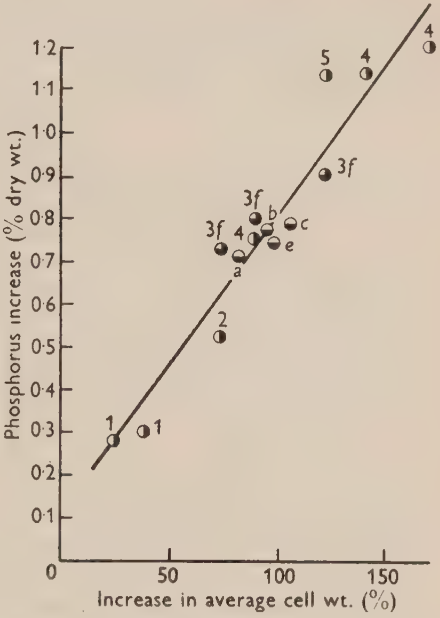

Fig. 8

Fig. 7. Comparison of growth rates and increase in total phosphorus content when half the first generation is formed during growth in CCY—the growth rate was varied by growing at different temperatures. Additional data refers to duration of the stationary phases at the respective growth rates. Temperatures: (*a*) 21°; (*b*) 23°; (*c*) 25°; (*d*) 30°; (*e*) 35°; (*f*) 37°; (*g*) 39·5°. A regression line was computed and is given.

Fig. 8. Comparison of increase in total phosphorus content and average cell weight when half the first generation is formed. The cultures had widely different growth rates, varied by employing different media at 37°, ◑ ◗ (numbers refer to the media listed in fig. 5) and by varying the temperature of growth in CCY cultures ◒ ◡ (letters refer to temperatures listed in Fig. 7). A regression line was computed and is given. It does not differ significantly from a line passing through the origin.

the increase in RNA takes at least until half the first generation to reach maximum—suggesting that the relationship is not a direct one. In addition, the evidence reported here suggests that the relationship does not hold when the growth rate is varied on the same medium by varying the temperature.

Working with *Esch. coli* and determining nucleic acid content from the ultra-violet absorption of the cells, Malmgren & Hedén (1947) found a direct relationship between the generation time and the amount of nucleic acid formed (Morse & Carter, 1949, later showed that RNA was mainly responsible). However, it did not hold true for the first generation. In the light of the evidence reported here this suggests that the bacterial cell requires a certain concentration of RNA, dependent upon its size, before it can divide. A plausible explanation of the temporary drop in RNA towards the end of the first generation (Figs. 4, 5) is that in the course of the first division part of the RNA phosphorus is metabolized into other phosphates or is lost from the cell.

I am pleased to acknowledge the assistance of Sgt. D. M. Morgan. I wish to thank Dr D. W. Henderson for his encouragement and advice and Dr D. Herbert and Major L. H. Kent for helpful criticism of this paper. The statistical treatment of results was carried out under the guidance of Mr S. Peto.

REFERENCES

Caldwell, P. C., Mackor, E. L. & Hinshelwood, C. (1950). The ribose nucleic acid content and cell growth of *Bact. lactis aerogenes*. *J. chem. Soc.* p. 3151.

Dawson, A. I. (1919). Bacterial variations induced by changes in the composition of culture media. *J. Bact.* **4**, 133.

Fiske, C. H. & SubbaRow, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375.

Gladstone, G. P. & Fildes, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

Leach, M. F. (1906). On the chemistry of *Bacillus coli communis*. *J. biol. Chem.* **1**, 463.

Malmgren, B. & Hedén, C. (1947). Studies of the nucleotide metabolism of bacteria. III. The nucleotide metabolism of the gram negative bacteria. *Acta path. microbiol. scand.* **24**, 448.

Morse, M. L. & Carter, C. E. (1949). The synthesis of nucleic acids in cultures of *Escherichia coli*, strains B and B/R. *J. Bact.* **58**, 317.

Schmidt, G. & Thannhauser, S. J. (1945). A method for the determination of desoxyribonucleic acid, ribonucleic acid and phospho-proteins in animal tissues. *J. biol. Chem.* **161**, 83.

Schneider, W. C. (1945). Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid of pentose nucleic acid. *J. biol. Chem.* **161**, 293.

Tracey, M. V. (1950). A colorimetric method for the determination of pentoses in the presence of hexoses and uronic acids. *Biochem. J.* **47**, 433.

Wade, H. E. (1952). Observations on the growth phases of *Escherichia coli*, American Type 'B'. *J. gen. Microbiol.* **7**, 18.

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TURNER-GRAFF, RUHAMA (1952). *J. gen. Microbiol.* 7, 31–35

An Effective Use of Petri Dishes for Microcultures

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SUMMARY: A microtechnique has been developed for the growth of many cultures in one Petri dish. The agar substrate is in the form of small plaques pipetted so that each plaque is distinct and separate from its neighbour in the Petri dish. The method is used for the assay of antifungal materials and has been adapted to the isolation and biochemical characterization of micro-organisms. Work is in progress to adapt the procedure to yeast and bacterial counts.

During work on the isolation and characterization of antibiotic substances, it was found essential to develop methods whereby the time of the investigator and the necessary materials were used more efficiently. Microtechniques appeared to hold the most promise. Microtechniques developed before and on the turn of the century have, for the most part, been neglected by later bacteriologists.

Koch (1881) was the first to recommend the use of slide cultures of semi-solid media for the microscopic observation of growth. The 'Little Plate Method' (Frost, 1921) is essentially a modification of Koch's technique. An accurate volume of milk (0·05 ml.) is mixed with a drop of agar on an accurately defined area on a sterile microscope slide. After incubation in a moist chamber for 4–16 hr., the area is dried and stained, and the colonies are counted under the microscope. In Burri's slant method (1928) an accurately shaped loop of a known diameter is dipped in milk and then run across the surface of an agar slant in a zig-zag manner so that the milk is spread evenly. After incubation, the colonies on the surface are counted.

Beijerinck (1889) introduced the auxanogram for the biochemical characterization of yeasts. The agar medium, heavily seeded with yeast, is poured into Petri dishes; when it is set, small amounts of the powdered test sugars are placed on the agar sufficiently far apart to prevent mixing when the powders have dissolved. Growth results in the sections where the utilizable sugar has diffused into the agar.

Lindner (1905) suggested a microtechnique for broth cultures. The substrate is inoculated with the test organism and drops of the resulting suspension are placed on a cover-glass by means of a sterile pen; the cover-glass is inverted on a hollow ground slide so that the hanging drops do not come in contact with the slide. Appropriate dilutions are made so that when observed microscopically, the growth developing from a single cell is seen. Henneberg (1934) adapted the procedure for his 'Federstrichkulture' technique. A broth medium is inoculated with the test organism and the suspension is streaked on a sterile cover-glass with a sterile pen point. Between streaks, the point is rinsed with uninoculated broth so that each successive streak is more dilute. The cover-glass is inverted on a hanging-drop slide and is sealed with a vaseline ring. After incubation, observation is made under the microscope.

Bronfenbrenner & Schlesinger (1918) described a micromethod for the identification of bacteria by fermentation tests; the substrate containing the agar, indicator and the sugar were deposited in droplets from a sterile capillary pipette in a Petri dish. These droplets, identified by markings on the outside of the Petri dish, were inoculated with the culture, and a piece of filter-paper soaked in 2 % NaOH was placed in the inverted Petri dish to absorb the CO_2 produced. This technique for the identification of coliform bacteria is very similar to the one developed independently in this laboratory, but it is the purpose of this paper to give the method a wider range of application.

The universally accepted cylinder plate method for antibiotic assay described by Heatley (1944) is essentially a modification of the auxanogram of Beijerinck. Small cylinders on an agar medium preseeded with the test organism are filled with the test solution. This diffuses through the agar and the resultant zones of inhibition are measured. The procedure to be described was an attempt to improve this method.

METHODS

Preparation of Petri dishes. Agar containing the nutrient materials and reagents or indicators to be used, is pipetted aseptically and falls as droplets on to the flat bottom of a Petri dish. The average volume of these droplets is 0·05 ml. and usually 2 droplets (0·1 ml.) are placed on the same site to cover an area of 1·0–1·2 cm. in diameter. Measured volumes of the agar may be pipetted when greater accuracy is desired. The agar solidifies almost immediately into circular plaques which are raised when the temperature of the agar droplets is close to the solidification point and flat when the temperature is higher. When comparisons are not quantitative, the agar may be poured directly from the test-tube containing the medium. The shape of the plaques may be varied at the time the droplets fall; an oval or narrow strip is obtained by placing 2 or 3 drops in a row.

The agar concentration in the original substrate is adjusted so that the solution will not be too fluid when the reagent under test is added. Generally a 2 % agar medium is used when the reagent is to be added up to 20 % concentration. When the reagent concentration is above this percentage, the agar in the original substrate is raised to 3 or 4 %.

With this technique, 8–30 plaques can be poured in each plate. As the agar solidifies almost immediately, the Petri dish may be inverted and agar plaques poured on the cover, so that the number of plaques is doubled.

The procedure may be adapted to the cylinder plate method of assay: the quantity of agar in each plaque is 0·2 ml. and the surface area correspondingly greater. A cylinder is firmly sealed in the centre of each plaque before the agar sets and the test solution is pipetted into the cylinder instead of being mixed with the substrate. The agar concentration used is 1·5 %.

Inoculation. The test organism may be inoculated in any of the following manners: (1) An active cell suspension is added to the agar before it is poured. (2) Each plaque is inoculated from an inoculating needle which has been dipped in a cell suspension of the test organism. (3) A sterilized cotton-wool ball, about 2 mm. in diameter, is dipped in a suspension of the test organism and then smeared evenly on the plaques so that the whole surface of each is covered. Sterile technique is used throughout.

This last procedure was found especially useful when the microcylinder plate technique was used. After the cylinder had been set in the agar and filled, the surrounding agar was smeared with the cell suspension.

Incubation. Inoculated Petri dishes are incubated at the optimal temperature of the test organism. For temperatures up to 37° the plaques retain sufficient moisture for the duration of the assay (up to 2 weeks at room temperature); above 37°, the plaques dry out before clear results can be obtained. For temperatures above 37° a beaker about 20 mm. in diameter and 8 mm. high, containing a small piece of cotton-wool, is partially sterilized in boiling water and then removed with flamed forceps. The excess water is poured off and after being flamed momentarily, the beaker is placed in the inoculated Petri dish on a drop of agar, which is melted by the heat of the beaker. When the agar sets it prevents movement of the beaker in the dish. It is not necessary to ensure absolute sterility of the beaker because it does not come in contact with any of the test plaques.

Cultures may be grown under anaerobic conditions with this technique, but evacuation of the jar will cause the plaques to dry out. The procedure can, nevertheless, be applied if the jar is not evacuated and the internal atmosphere is changed without a reduction in gas pressure. Partial reduction of O_2 tension is obtained if the inoculation is made in the liquid agar before the plaques are poured, or a drop of agar or a sterile cover-glass may be used to cover the inoculated plaque.

The inhibitory or stimulatory action of the test solutions may be determined by noting the incubation time necessary for visible growth to appear and by comparing the colony size with that of a control. Where indicators are added, the change in the indicator is clearly seen. In the cylinder cup application, the zone of inhibition is measured.

Application to the assay of antifungal substances

Wort agar at pH 7·0 is mixed with the material under test (pH 5–7) in varying proportions so that its final concentration varies from 1 to 50 %. The agar concentration is adjusted as previously described, and is further increased by 25 % when the final pH value is 5·5 as the agar is partly hydrolysed at this pH.

When the plaques are set they are labelled by symbols on the outside of the Petri dish. Identification may be made with a glass-marking pencil or indian ink, but it was found more convenient to prepare written adhesive labels before the plates were poured since these could be glued to the plates without inverting them.

The plaques are now inoculated from a spore suspension of *Penicillium roqueforti* with an inoculating needle. Incubation is at 25°. Preliminary results may be noted after 48 hr. incubation but after 72 or 96 hr. results are much clearer. Pl. 1 shows the appearance of inoculated and uninoculated plaques after incubation.

The volume of substrate and test solution is 1–2 ml. Plaques are poured in duplicate or triplicate and the agar remaining in a small test-tube is inoculated with an inoculating needle. The test tube culture serves as a check of the results obtained with the plaques. The microcylinder cup technique is occasionally used as an additional check.

When the antagonism between two organisms is being determined, the plaques are poured in an elongated shape (two or three drops of agar in a row). The two organisms are inoculated at opposite ends of the plaque and the resulting antagonism may be determined after incubation.

DISCUSSION

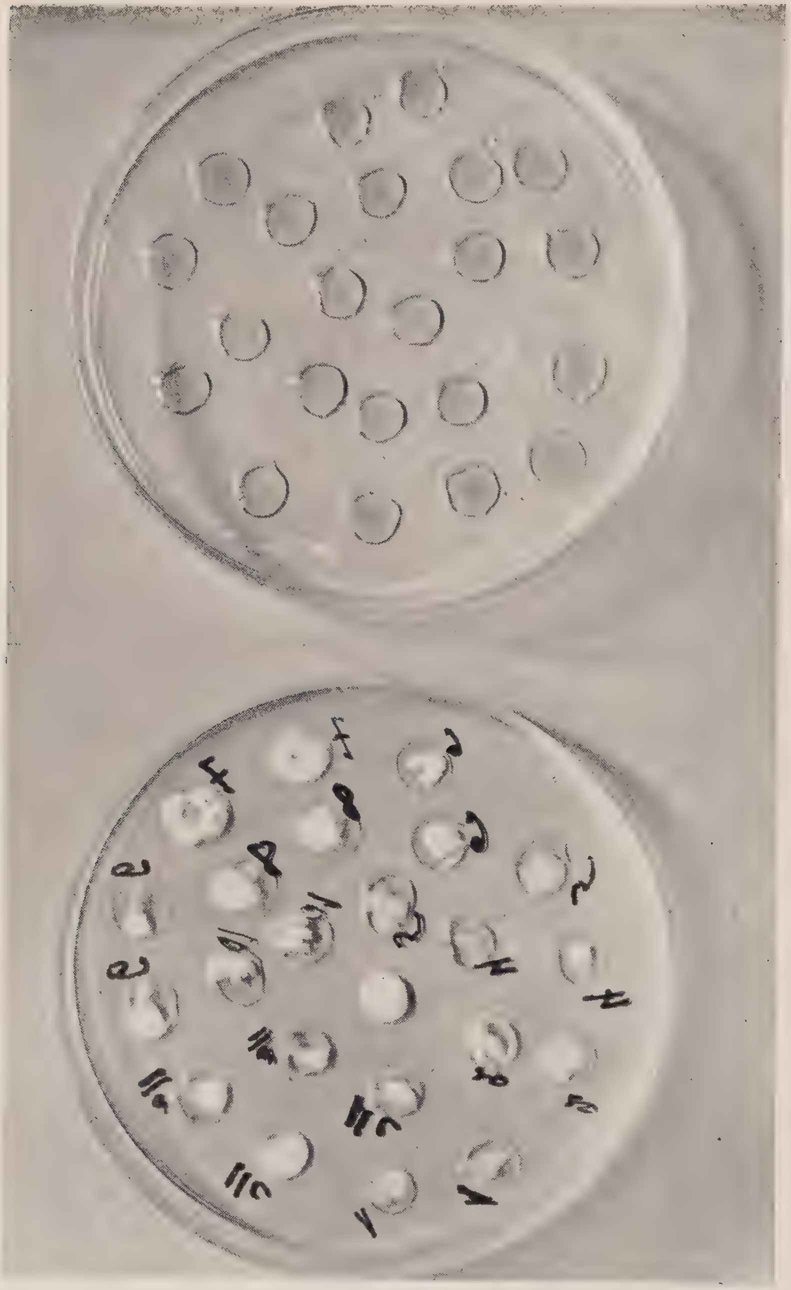
Several drawbacks are evident if the microtechniques described by earlier authors are critically examined. The slide-cells used by Koch (1881), Frost (1921), Lindner (1905) and Henneberg (1934) require great care and accuracy, and it is doubtful whether complete sterility is maintained. Special moist chamber incubators are often required and the procedure as a whole is not adaptable to routine work with many samples. In the Burri (1928) procedure, only one inoculation can be made per tube and only aerobic organisms develop.

In the auxanogram of Beijerinck (1889) and in the cylinder plate method for antibiotic assay, diffusion through the agar may reach such proportions that the results in one segment are completely obscured by an adjoining segment. In addition, spreading organisms may cover the whole surface of the agar and completely obscure results. Finally, relatively large volumes of the substrate are used.

The microtechnique advocated in this paper has the following advantages: (1) Time, space on tables and in incubators, dishes and substrate material are saved. (2) All the manipulations can be made by one worker except in the microcylinder cup assay, for which assistance is needed to insert the cylinder before the agar has solidified. (3) The results are concentrated in a small area so that the examination of the growth is more distinct. (4) The agar solidifies immediately and inoculation can be made at once. (5) The danger of condensation water dropping on to the agar is virtually eliminated. (6) Each plaque is distinct from its neighbour so that the results on one are not influenced by any other. (7) The danger of air contamination is lessened (less nutrient area).

The technique of smearing the plaque with cotton-wool assures an even inoculation without the danger of breaking the surface of the agar. A sterile glass rod or sterile brush are not as satisfactory because the former cools only slowly after flaming and the latter can only be used once.

When this paper was in preparation, Dingle & Solomons (1951) published a micromethod for measuring the biochemical activities of micro-organisms. The organism is grown in small cylinders of agar. The culture is then extruded with a glass rod and the test agar medium is added to enclose completely the agar plugs. The biochemical activity can then be determined with suitable developing agents in the agar. The manipulations are more complicated than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Ruhama Turner-Graff—Microcultures in petri dishes. Plate 1 the method proposed here. Since the plate is flooded with agar for biochemical tests, it has the drawbacks of the auxanogram of Beijerinck.

The method described can be used for semi-quantitative biochemical work in bacteriology, especially where the reagent under test is only available in small quantities or is expensive. The method is also of great advantage where a series of determinations is made, and it greatly simplifies isolation experiments. Work is in progress to adapt the technique to bacterial and yeast counts.

Appreciation is expressed to Dr J. Babad, head of the Laboratory, for facilities provided, and to Dr A. Pinsky for assistance in preparing the manuscript.

REFERENCES

Beijerinck, M. W. (1889). L'auxanographie ou la méthode de l'hydrodiffusion dans la gélatine appliquée aux recherches microbiologiques. *Arch. Nederland*, 23, 367. Cited from Küster, E. (1921), *Kultur der Mikroorganismen*, p. 97. Leipzig: B. C. Teubner.

Bronfenbrenner, J. & Schlesinger, M. J. (1918). A rapid method for the identification of bacteria fermenting carbohydrates. *Amer. J. publ. Hlth*, 8, 922. Cited from *Chem. Abstr.* (1919), 13, 968.

Burri, R. (1928). The quantitative milk culture. A simple means for the bacteriological examination of milk. *Proc. World's Dairy Congr. London*, p. 690. Cited from *Standard Methods for the Examination of Dairy Products* (1948), 9th ed., p. 339. New York: American Public Health Association.

Dingle, S. & Solomons, G. L. (1951). A micro-method for the examination of the biochemical activities of micro-organisms. *Nature, Lond.* 168, 425.

Frost, W. D. (1921). Improved technic for the micro or little plate method of counting bacteria in milk. *J. infect. Dis.* 28, 176. Cited from F. W. Tanner, (1944), *The Microbiology of Foods*, 2nd ed., p. 355. Garard Press, Champaign, Ill., U.S.A.

Heatley, N. G. (1944). A method for the assay of penicillin. *Biochem. J.* 38, 61.

Henneberg, W. (1934). Herstellung der Federstrichkulturen. *Bakteriologische Molkereikontrolle*, p. 3. Berlin: Paul Parey.

Koch, R. (1881). Cited from Küster, E. (1921), *Kultur der Mikroorganismen*, p. 53. Leipzig: B. C. Teubner.

Lindner (1905). *Mikrosk. Betriebskontrolle in der Gärungsgewerben.* 4th ed. Berlin. Cited from Küster, E. (1921). *Kultur det Mikroorganismen*, p. 57. Leipzig: B. C. Teubner.

EXPLANATION OF PLATE

Appearance of Petri dishes after incubation. Upper: uninoculated plaques; lower: microcultures.

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Some Factors affecting the Viability of Faecal Bacteria in Water

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SUMMARY: The effect of various conditions on the rate of death of *Bacterium coli* and of *Streptococcus faecalis* in dilute buffer solutions was followed by making colony counts at intervals during prolonged periods of incubation and then constructing mortality curves. The form of these curves was characteristic for each organism. Of the pH values investigated (approximately 5, 6, 7 and 8) both organisms were least viable in the range 6 to 7. *Strep. faecalis* was not affected by the degree of oxygenation of the water but *Bact. coli* died much more rapidly under anaerobic than under aerobic conditions.

Growth of *Bact. coli* was observed with as little as 0·28 part per million organic matter in solution. A much higher concentration of nutriment was required for growth of *Strep. faecalis*; with a concentration slightly below that required for growth the viable population remained virtually stable for a long period. The rate of death depended on the age of the cells at the time of immersion in water.

Factors affecting the longevity in water of organisms used as indices of pollution are of importance in the sanitary survey of rivers or in assessing the suitability of water for drinking purposes. Reports in the literature suggest that the period of survival may be conditioned by a number of factors but the conclusions drawn are often open to doubt owing to failure to arrange for conditions to be the same in comparable experiments. Moreover, in earlier work there is often some doubt of the precise characters of the organism referred to as *Bacterium coli*. Very few observations appear to have been made on the longevity of *Streptococcus faecalis*.

METHODS

The factors studied were pH value, oxygen tension, concentration of nutrient material, and age of culture at time of immersion in water. In the investigation of any one factor, precautions were taken to ensure that other conditions remained constant. The strains of bacteria used had all been isolated from sewage or river water and before use in this work were tested for characteristic reactions. These were: *Bact. coli* (type I): Gram negative, non-sporing short rod, indole +, MR +, VP −, citrate −, acid and gas in MacConkey broth at 44°; *Strep. faecalis*: Gram positive coccus in pairs and short chains, acid clot in litmus milk, growth at 45°, growth in broth containing 6·5 % sodium chloride, survives heating for 30 min. at 60°, growth at pH 9·6. Incubation was at 20° unless otherwise stated.

Very dilute phosphate buffer solution (0·003 M) was used as the suspending fluid, a concentration sufficient to maintain the pH value virtually constant throughout the course of an experiment. The solution was sterilized by autoclaving either in hard glass bottles fitted with stoppers or in conical flasks covered with beakers; cotton wool or other organic material was not allowed in contact with the buffer or with the inside of a flask. Unless anaerobic conditions were being studied the solution was aerated with sterile diffused air for 1 hr. before use.

Colony counts were made either in Petri dishes or by the spinning bottle technique described by Proom & Hemmons (1949). Nutrient agar was used as the medium for *Bact. coli*, glucose yeast extract agar for *Strep. faecalis*; inoculated plates and bottles of both media were incubated at 37°.

RESULTS

Form of death curve

When numbers of surviving bacteria, expressed as percentages, were plotted against the periods for which the culture had been suspended in buffer, the form of the curve was usually sigmoid, as in Fig. 1. Such curves approached the horizontal axis so gradually that an exact period of survival could not be estimated. When, however, the percentages of bacteria surviving were replaced by the logarithms of the percentages, the curves approached the horizontal axis much more steeply, as in Fig. 2. The period of survival was therefore conveniently expressed as the time corresponding to the point of intersection of such a curve with the axis, i.e. the time required for destruction of 99 % of the cells originally present.

The curves for *Bact. coli* and *Strep. faecalis* were characteristically of different shapes, and corresponded to the two principal types of deviation from the logarithmic form discussed by Hinshelwood (1951).

With *Bact. coli* a large section of the 'log-survivors' curve sometimes approximated to a straight line, as in Fig. 2 (*Bact. coli*), but often the final stages of decline might be abnormally protracted so that the curve turned outwards at the lower end, as in Fig. 3 and in curve *A*, Fig. 5. This could be explained by the presence of cells of abnormally high resistance, which Hinshelwood suggests may arise from a previous mutation. Some support for this view was obtained in one set of experiments where the deviation was particularly marked and plate cultures of the declining population showed the presence of both smooth and rough forms of colony, both of which when tested gave characteristic reactions for *Bact. coli*. This variation was, however, only transient for, on preparing cultures from the two types of colony and comparing their rates of decline when suspended in dilute buffer solution, both the 'log-survivors' curves were found to approximate closely to straight lines and to show very similar rates of decline. Platings from both these suspensions now showed the presence of only smooth colonies.

With *Strep. faecalis* some time elapsed before the lethal action of the water took full effect but then it acted comparatively rapidly. The 'log-survivors' curve sloped very gently at first and then quite steeply. In some tests there was no appreciable diminution in count for several days and then in a further similar period of time the suspension was virtually totally destroyed.

Effect of pH *value*

Rates of death were determined at pH values approximately 5, 6, 7 and 8, in buffer solutions containing 5 parts per million (p.p.m.) of an 18 hr. nutrient broth culture of *Bact. coli* or 3 p.p.m. of a 72 hr. culture of *Strep. faecalis* in glucose-yeast extract broth. Fourfold colony counts were made on the contents of duplicate flasks at intervals during incubation. Rates of death in duplicate flasks were found to be of the same order, but there were profound differences between average rates of death at different pH values. Fig. 1, in which the averages of the eight values are plotted, shows the same trend for both organisms; viability is greatest at pH 5·0–5·2, least at pH 6·1–7·1 and intermediate between these extremes at pH 7·6–7·7.

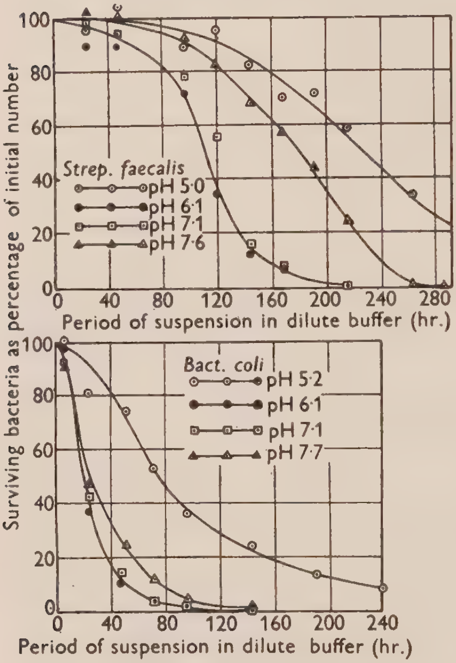

Fig. 1

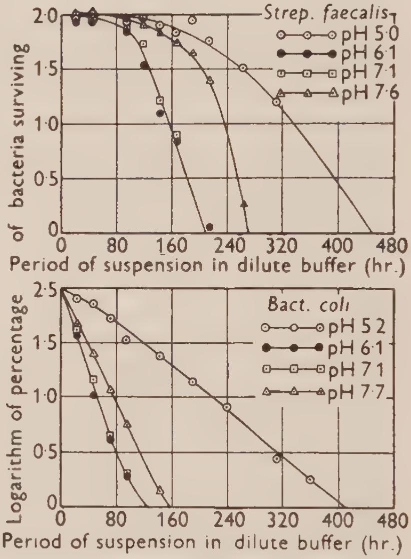

Fig. 2

Fig. 1. **Rate of death of *Bact. coli* and of *Strep. faecalis* in 0·003 M phosphate buffer at different pH values.**

Fig. 2. **Rate of death of *Bact. coli* and of *Strep. faecalis* in 9·003 M phosphate buffer at different pH values (logarithmic scale).**

Effect of oxygen tension

Rates of death of each organism under aerobic and anaerobic conditions were compared. For several hours before use a current of sterile nitrogen was passed through the liquid in the bottles to be used for anaerobic conditions, anaerobiosis being confirmed by testing for dissolved oxygen by the micro method of Fox & Wingfield (1938). The buffer for aerobic experiments was aerated with a current of sterile air. Four bottles (two aerobic and two anaerobic) were each inoculated with 5 p.p.m. of an 18 hr. broth culture of the test organism. Before and immediately after each sampling a current of air or nitrogen was passed through the suspensions and the bottles were then resealed. In all aerobic bottles the liquid remained virtually saturated throughout the experiments. In the anaerobic bottles containing *Bact. coli* the dissolved oxygen remained between 1·5 and 4·0 % of saturation for the first 3 days and between 9 and 18 % for the next 6 days; in those inoculated with *Strep. faecalis* it was between 1 and 6 % of saturation during the first 6 days of incubation, after which no further values were obtained.

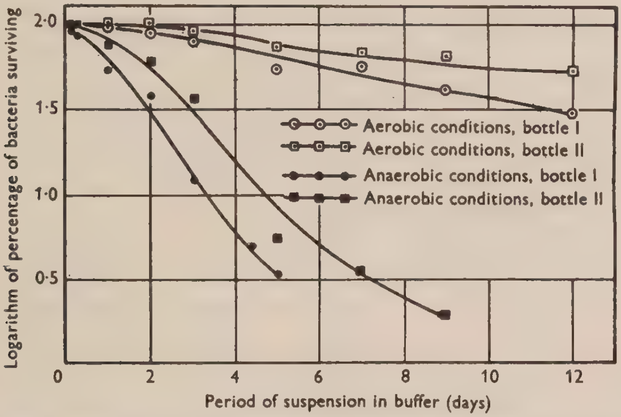

Fig. 3. Rate of death of *Bact. coli* in 0·003 M phosphate buffer at pH 6·0 under aerobic and anaerobic conditions.

Results (expressed graphically in Figs. 3 and 4) show that *Bact. coli* died much more rapidly under anaerobic than under aerobic conditions, a finding similar to that observed by Whipple & Mayer (1906) for *Salmonella typhi*. With *Strep. faecalis*, on the other hand, there was no significant difference in the rates of mortality under the two conditions.

Effect of small concentrations of nutrient material

Reports in the literature suggest that growth of bacteria in water containing only traces of nutrient material may depend on a number of conditions. Savage & Wood (1917) found that, whereas streptococci did not multiply in sterile tap water, *Bact. coli* showed an increase in every test. Butterfield (1929) recorded growth of *Bact. aerogenes* in media containing 0·5 p.p.m. glucose and 0·5 p.p.m. peptone, ZoBell & Grant (1943) of heterotrophic marine bacteria in mineral solutions containing as little as 0·1 p.p.m. peptone or glucose, and Burke & Taschner (1936) of *Bact. coli* in water containing sufficient potato extract to give a figure for ‘oxygen consumed’ of 0·51 p.p.m. or above. Heukelekian & Heller (1940) found that by increasing the effective surface (for example by adding glass beads) appreciable growth of *Bact. coli* was obtained at much lower concentrations of nutrient material than normally. This effect of surface was thought by ZoBell (1943) to be largely due to concentration of nutrient material on it. Bigger (1937) observed that one strain of *Bact. coli* attained numbers of several hundred thousand/ml. in sterilized tap water and over a prolonged period of storage the population showed pronounced rises and falls. Further experiments (Bigger & Nelson, 1941, 1943) showed that, provided ammonia and carbon dioxide were present, *Bact. coli* would grow in distilled water containing talc or one of a number of other inorganic materials in a finely divided or porous condition.

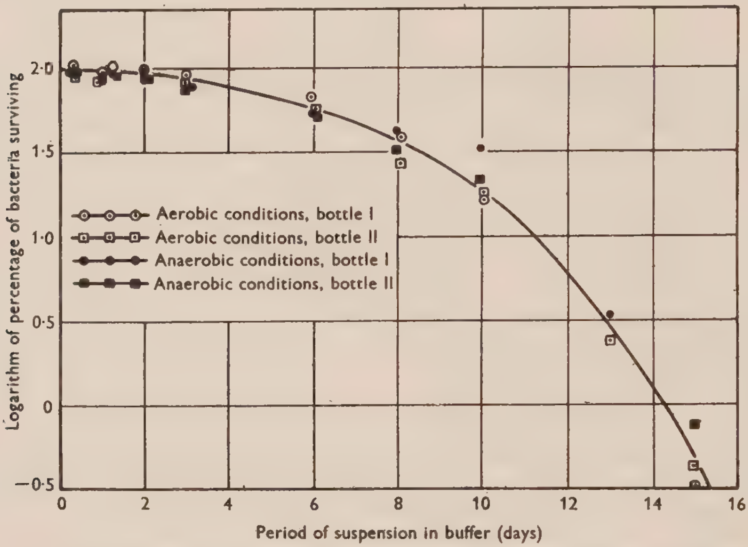

Fig. 4. Rate of death of *Strep. faecalis* in 0·003 M phosphate buffer at pH 6·0 under aerobic and anaerobic conditions.

Observations on the effect of small concentrations of nutrient material on viability were made with suspensions of organisms which had been freed as far as possible from organic matter by repeatedly centrifuging and resuspending in buffer solution. Measured quantities of appropriate dilutions of glucose-yeast extract broth (0·3 % Yeastrel, 2 % peptone, 0·5 % glucose, and 1 % Lemco) were added to separate bottles of the bacterial suspension to give concentrations ranging from nil to several hundred p.p.m.

Two sets of experiments were carried out with each species of organism, the first with a pure culture of a single strain, the second with a mixture of several strains. Freedom from contamination was established periodically throughout the experiments by picking colonies from the plates and testing their characters.

The average counts recorded in these experiments are shown in Tables 1 and 2. Growth of the mixed strains of *Bact. coli* occurred at much lower concentrations of nutrient material than with the single strain and the level of population eventually maintained increased with the concentration of nutriment. The lowest concentration of broth in which substantial growth occurred

Table 1. *Effect of small concentrations of nutrient material on viability of* Bact. coli *in* 0·003 M *phosphate buffer at* pH 6·0.

(Surviving bacteria as colony counts/ml. of suspension, each figure being the average of three counts.)

Period of suspension in buffer (hr.)	Concentration of added broth (p.p.m.)					
	0	2·5	10	25	100	250
	Colony counts/ml. suspension					
Exp. I. Pure culture of single strain:						
0	2,340	2,280	—	2,390	—	2,080
23	900	299	—	290	—	> 20,000
44	374	101	—	457	—	> 20,000
71	193	81	—	585	—	> 20,000
95	131	61	—	548	—	6,200,000
143	81	46	—	356	—	100,000,000
240	34	26	—	37	—	7,400,000
336	27	14	—	9	—	2,980,000
432	20	8	—	4	—	3,550,000
504	9	2	—	2	—	2,740,000
583	4	0	—	0	—	2,790,000
672	1	0	—	0	—	3,810,000
744	0	0	—	0	—	4,010,000
Exp. II. Mixed culture of five strains:						
0	1,720	1,880	1,260	1,580	1,950	1,900
22	401	151	279	520	72,000	850,000
47	274	80	278	1,370	1,860,000	8,800,000
71	129	69	584	6,430	49,600,000	522,000,000
94	89	105	2,000	40,000	4,000,000,000	4,000,000,000
166	86	57	> 5,000	107,000	1,220,000	3,290,000
214	97	57	67,000	160,000	1,190,000	6,700,000
264	65	42	56,000	79,000	800,000	2,800,000
336	55	42	44,000	83,000	850,000	2,850,000
415	58	39	50,000	83,000	680,000	2,610,000
504	61	56	53,000	104,000	950,000	2,690,000
576	52	39	49,000	70,000	660,000	2,730,000

was 10 p.p.m., corresponding to a content of 0·03 p.p.m. Yeastrel, 0·2 p.p.m. peptone, 0·05 p.p.m. glucose, and 0·1 p.p.m. Lemco. The 5-day biochemical oxygen demand (at 18·3°) of this dilute broth was 0·26 p.p.m. and the oxygen absorbed from acid permanganate in 4 hr. at 26·7° was 0·08 p.p.m.

With streptococci no growth was observed in any of the concentrations of broth up to 375 p.p.m. though the period of survival tended to be prolonged as the concentration increased. With 375 p.p.m. broth, survival was so prolonged that there was virtually no diminution in numbers over the test period of 34 days. When the concentration of nutriment rose to 500 p.p.m. slight but definite growth took place. This was 50 times the lowest concentration in which growth of *Bact. coli* had been observed.

The presence of a minute quantity of organic matter (less than 1 p.p.m.) may induce a profound departure from the true form of the death curve for *Bact. coli* (Fig. 5).

Table 2. *Effect of small concentrations of nutrient material on viability of* Strep. faecalis *in* 0·003 M *phosphate buffer at* pH 6·0

(Surviving bacteria as colony counts/ml. of suspension.)

Period of suspension in buffer (hr.)	Concentration of added broth (p.p.m.)			
	0	3·75	37·5	375
	Colony count/ml. suspension			
Exp. I. Pure culture of single strain (average results of eight counts):				
0	3240	3190	3360	3230
5	3530	3520	3390	3530
24	3050	3300	3380	3370
75	—	—	3160	3560
97	286	1770	—	—
120	173	426	2990	3590
147	39	59	1770	3400
168	—	—	893	3600
172	3	7	—	—
190	—	—	501	3170
243	—	—	177	3350
268	—	—	72	—
292	—	—	47	3370
34 days	—	—	—	2880

Period of suspension in buffer (hr.)	Concentration of added broth (p.p.m.)					
	0	10	25	100	250	500
	Colony count/ml. suspension					
Exp. II. Mixed culture of six strains (average results of four counts):						
0	460	427	457	453	557	440
24	214	229	200	237	356	334
72	49	57	50	86	203	319
96	32	33	41	49	164	460
124	19	29	20	32	133	700
163	9	15	13	20	99	640
192	4	10	9	16	81	593
244	3	7	6	15	79	623
264	—	—	—	—	77	1573

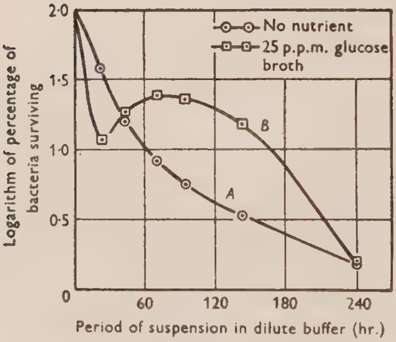

Fig. 5

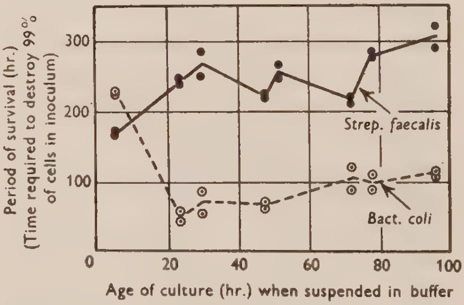

Fig. 6

Fig. 5. Effect of trace of nutrient material on rate of death of *Bact. coli* in 0·003 M phosphate buffer.

Fig. 6. Periods of survival in 0·003 M phosphate buffer at pH 6·0 of cultures of *Bact. coli* and *Strep. faecalis* of different ages.

Effect of age of cells at time of immersion

At intervals during incubation (at 37°) of broth cultures of the test organisms dilute suspensions were prepared in phosphate buffer at pH 6·0, each suspension containing approximately 5 p.p.m. of culture.

The periods of immersion required to destroy 99 % of bacteria are shown for each pair of duplicate bottles in Fig. 6. It is evident that age affected the two species differently, a young culture of *Bact. coli* being more resistant, and a young culture of *Strep. faecalis* more sensitive, than older cultures. The period of survival of both organisms tended to increase as the culture aged between 24 and 96 hr. With *Strep. faecalis* this tendency was accompanied by fluctuations in resistance; these were less marked with *Bact. coli.*

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REFERENCES

Bigger, J. W. (1937). The growth of coliform bacilli in water. *J. Path. Bact.* **44**, 167.

Bigger, J. W. & Nelson, J. H. (1941). The growth of coliform bacilli in distilled water. *J. Path Bact.* **53**, 189.

Bigger, J. W. & Nelson, J. H. (1943). The metabolism of coliform bacilli in distilled water. *J. Path. Bact.* **55**, 321.

Burke, V. & Taschner, L. (1936). Growth of *Escherichia coli* in a small amount of organic matter. *J. Bact.* **32**, 124.

Butterfield, C. T. (1929). Experimental studies of natural purification in polluted waters. III. A note on the relation between food concentration in liquid media and bacterial growth. *Publ. Hlth Rep., Wash.* **44**, 2865.

Fox, H. M. & Wingfield, C. H. (1938). A portable apparatus for the determination of oxygen dissolved in a small volume of water. *J. exp. Biol.* **15**, 437.

Heukelekian, H. & Heller, A. (1940). Relation between food concentration and surface for bacterial growth. *J. Bact.* **40**, 547.

Hinshelwood, C. (1951). Decline and death of bacterial populations. *Nature, Lond.* **167**, 666.

Proom, H. & Hemmons, L. M. (1949). The drying and preservation of bacterial cultures. *J. gen. Microbiol.* **3**, 7.

Savage, W. G. & Wood, D. R. (1917). The vitality and viability of streptococci in water. *J. Hyg., Camb.* **16**, 227.

Whipple, G. C. & Mayer, A. (1906). On the relation between oxygen in water and the longevity of the typhoid bacillus. *J. infect. Dis.* (Suppl. no. 2), 76.

ZoBell, C. E. (1943). The effect of solid surfaces upon bacterial activity. *J. Bact.* **46**, 39.

ZoBell, C. E. & Grant, C. W. (1943). Bacterial utilization of low concentrations of organic matter. *J. Bact.* **45**, 555.

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On the Microbiological Assay of Threonine

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SUMMARY: A small lag section has been noticed in the standard curves obtained in the estimation of threonine using *Streptococcus faecalis R*. An increase in the acetate and phosphate buffers in the basal medium has been found to be advantageous.

While investigating the amino-acid composition of some Indian foods by the microbiological method, the authors made certain observations concerning the assay of threonine with *Streptococcus faecalis R* to which little attention has been paid in the literature. Barton-Wright (1946), Horn, Jones & Blum (1947) and several other workers have employed *Strep. faecalis R* for the assay of this amino-acid.

An initial lag section was observed in the standard threonine curves when *Strep. faecalis R* was used as the test organism. Certain minor improvements to the basal medium have also been made to secure a good response over a wide range of the amino-acid.

EXPERIMENTAL

Strep. faecalis R (ATCC 9790) was used throughout these studies.

The basal media and the method of Barton-Wright (1946), with certain minor modifications, have been successfully employed in this laboratory for the routine assay of the various amino-acids in foods. The final volume was 5 ml./tube, made up of 2·5 ml. basal medium and 2·5 ml. standard solution and water. This reduction in volume was mainly effected so as to economize the use of the costly synthetic amino-acids. Xanthine was not available and hence was omitted from the media. The concentration of amino-acids and other ingredients in the media was the same as recommended by Barton-Wright (1946).

When threonine was determined by the above method, the acid production was rather low and the range of the standard curve was also limited (6 ml. 0·025 N acid for a range of 0–20 μg. of DL-threonine) even after making due allowances for the reduced final volume/tube. An increase in the concentration of glucose and sodium acetate alone had been found to be without effect on the acid production (Barton-Wright, 1946).

A survey of the literature revealed that sodium citrate had a high buffering action and allowed better growth and acid-production than sodium acetate for *Strep. faecalis* (Teply & Elvehjem, 1945; Rabinowitz & Snell, 1947). Hence, the search for a medium which contained sodium citrate as a buffer resulted in the media and the method of Sauberlich & Baumann (1946). A trial was made with this method. However, it was found that this method also suffered from several disadvantages. The end-point with bromothymol blue in the final titrations of the acid produced was not sharp, the colour change being imperceptibly slow even after the addition of an excess of 0·5–1·0 ml. alkali. Moreover, the acid production was also low. (5 ml. 0·025 N acid for a range of 0–20 μg. of DL-threonine). When sodium citrate was replaced by sodium acetate in the medium, the end-points were sharp, but the acid production and range of the curve continued to be as before. Further, when the medium with sodium acetate was used to assay the threonine content of foods, erratic values were obtained. There was no agreement among the values calculated at different assay levels, the standard curves were not superimposable, and the values were not reproducible.

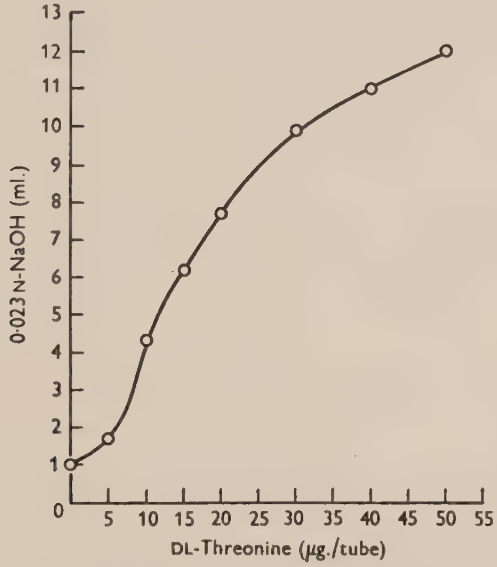

Fig. 1. A typical standard curve obtained in the assay of threonine employing *Strep. faecalis R.*

An attempt was therefore made to use the original method of Barton-Wright (1946) itself, with certain additional modifications. The buffering capacity of the medium was increased by doubling the concentration of sodium acetate and of the phosphates. The amount of glucose was also doubled, since in the presence of increased buffers greater acid production might be possible with more glucose. Satisfactory end-points and good acid production were obtained. (11 ml. 0·025 N acid for a range of 0–50 μg. of DL-threonine.) Values obtained by the use of this modified medium on foods were reliable on the basis of the various criteria for dependability of microbiological assays.

A typical standard curve obtained in the assay of threonine with the modified medium is shown in Fig. 1.

DISCUSSION

It may be seen from the standard curve that there is an initial lag section in the region 0–5 μg. DL-threonine and that maximum acid production occurs at 50 μg. of the DL-acid, after which the curve becomes parallel to the abscissa. Horn *et al.* (1947) have reported a response up to 200 μg. DL-threonine, with 10 ml. as the final volume/tube. However, though the same organism from the same source was used in the present case, the response and the range were not as high as could be expected, even after making due allowance for the restricted final volume employed.

Meinke & Holland (1948), while studying the inhibition of serine utilization by threonine in some lactic acid bacteria, made an incidental observation that there was also an inhibition of threonine utilization by serine. Although no detailed figures were given, they concluded that a ratio of serine to threonine of less than 200:1 for *Strep. faecalis R* was necessary for the successful utilization of threonine in presence of serine. This may possibly explain some of our observations. In the present modified medium, DL-serine is present to the extent of 1 mg./tube. Hence, it can be expected that when *Strep. faecalis R* is used as the organism, 1 mg./200, i.e. at least 5 μg. threonine, must be present to circumvent the inhibitory effect of serine. The lag section in the standard threonine curves of the authors can, therefore, be attributed to some extent to the presence of serine in the medium at the level indicated.

In the case of the workers who developed curves up to 200 μg. threonine, it is possible that this small lag section was overlooked.

The implications of this lag section on the interpretation of the curve are quite obvious. In addition to the serine present in the medium, a small amount of serine is likely to be provided by the experimental hydrolysates. A measure by which the effect of added serine can be made insignificant is to have a sufficiently large amount of serine in the medium itself. The present modified medium satisfies such a condition since the amount of serine from the medium will be 1 mg./tube and any serine contributed by the hydrolysates may be only of the order of 50 μg.—a variation of only 5 % in the total serine content.

The levels of hydrolysate should be conveniently chosen so that readings are obtained between 10 and 40 μg. DL-threonine and the lag section of the curve is avoided. Such a procedure has been found to give highly reliable and reproducible values.

The values for the threonine content of some Indian foods using the modified medium will be reported elsewhere in due course.

The authors are indebted to Dr S. S. De for valuable advice and helpful criticism, to Dr K. V. Giri for his keen interest, to Dr M. Sirsi for kind help in maintenance of cultures and to the Indian Council of Medical Research for generous financial assistance.

REFERENCES

Barton-Wright, E. C. (1946). The microbiological assay of amino-acids. I. The assay of tryptophan, leucine, isoleucine, valine, cystine, methionine, lysine, phenylalanine, histidine, arginine and threonine. *Analyst*, **71**, 267.

Horn, M. J., Jones, D. B. & Blum, A. E. (1947). Microbiological determination of threonine in proteins and foods. *J. biol. Chem.* **169**, 739.

Meinke, W. W. & Holland, B. R. (1948). Threonine-serine antagonism in some lactic acid bacteria. *J. biol. Chem.* **173**, 535.

Rabinowitz, J. C. & Snell, E. E. (1947). The vitamin B_6 group. XI. An improved method for assay of vitamin B_6 with *Streptococcus faecalis*. *J. biol. Chem.* **169**, 631.

Sauberlich, H. E. & Baumann, C. A. (1946). The effect of dietary protein upon amino-acid excretion by rats and mice. *J. biol. Chem.* **166**, 417.

Teply, L. J. & Elvehjem, C. A. (1945). The titrimetric determination of '*Lactobacillus casei* factor' and 'folic acid'. *J. biol. Chem.* **157**, 303.

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Toxin Production in Two Strains of Staphylococcus and their Variants

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SUMMARY: Two mouse virulent strains of *Staphylococcus aureus* and their avirulent variants were tested for their resistance to the bactericidal power of fresh human blood and for toxin production.

The variants were much more easily killed by fresh blood than the parent strains and had lost the ability to produce α-haemolysin, dermonecrotic toxin and leucocidin.

The Panton-Valentine leucocidin test was supplemented by two others that gave more reliable results, one depending on direct observation of leucocytes emigrated from a clot on to the surface of a slide, the other on the destruction of bactericidal power of the leucocytes.

Avirulent variants obtained from a virulent strain of *Staphylococcus aureus* by exposure to noxious agents for a sublethal time have been described (Voureka, 1952). In these variants the loss of virulence was always accompanied by loss of certain characters connected with virulence such as coagulase production, mannitol fermentation and gelatin liquefaction.

It was of interest to investigate other characters associated with virulence in parent and variant strains. Resistance to the bactericidal effect of whole blood, haemolysis, dermonecrotic effect and leucocidin were tested.

Two strains of staphylococci, A and B, were used. The parent strain A, originally obtained from a human infection, had been kept on artificial media for 4 years; and at the time of its use was rough in colonial appearance although highly virulent for mice. Of its variants AIII was obtained by exposing A to 20μg. chloramphenicol/ml. for 24 hr. *in vitro*. AVIII was similarly obtained using 50μg. terramycin/ml. (Voureka, 1952). A10 was obtained from the heart blood of a mouse infected intraperitoneally with A and given 2·5 mg. terramycin intravenously 12 hr. later.

Parent strain B was freshly isolated from a human infection and was smooth and mouse virulent. Its variant B4 was obtained in a similar way to A10.

Resistance to bactericidal effect of blood

Organisms vary greatly in their resistance to the bactericidal action of fresh human blood, and virulent strains usually are much more resistant than avirulent strains. Resistance to bactericidal power was tested by the slide cell technique (Wright, 1923). Dilutions of 16 hr. broth cultures of the staphylococcal strains were made and a sample of each dilution was taken for viable counting in serum in slide cells. At the same time 25 cu.mm. of normal human blood and 5 cu.mm. of each culture dilution were mixed and run into slide cells. After overnight incubation at 37° the number of colonies developing in the slide cells were counted. The approximate number of staphylococci added to each slide cell was estimated from the viable count. The results obtained are shown in Table 1. The greater bactericidal power of the blood for the variants than for the parent strains is clearly demonstrated.

Table 1. *Bactericidal effect of blood on staphylococcal strains*

Strain of staphylococcus	Approximate number of staphylococci added	Number of colonies developing in slide cells
A	2600	369
AIII	2900	12
AVIII	6500	50
A10	2900	22
B	2250	246
B4	2500	7

Toxin-antitoxin precipitation in solid media

The method used was similar to that described by Elek (1948). A $\frac{1}{4}$ in. gutter in an agar plate was filled with agar containing a high concentration of staphylococcal antitoxin (refined staphylococcus antitoxin globulins, Burroughs Wellcome). Streaks of the staphylococci were made at right angles to this gutter and the plate incubated at 37° in 20 % CO_2 for 48 hr. or longer. Lines of precipitate were formed (Pl. 1, fig. 1). They are numerous and clearly seen near the streaks of A and B. A10, AVIII and B4 each showed two very indistinct lines which are scarcely visible in the photograph. AIII formed one very poorly visible line.

Methods of obtaining and testing toxins

Haemolysin, dermonecrotic factor and leucocidin. The organisms were seeded on plates of 0·5 % nutrient agar. These were incubated at 37° in 20 % CO_2. An uninoculated plate of 0·5 % agar treated in the same way served as a control.

After incubation the agar was centrifuged. The supernatant from 6-day cultures was used directly for haemolysin and dermonecrotic toxin tests, and that from 24 hr. cultures was filtered through a collodion membrane to ensure sterility in tests for leucocidin. Parent and variant strains showed good growth in all cases.

Haemolysins. (*a*) *Tube tests.* The supernatant was used unheated and after heating to 55° for 30 min. to destroy α-haemolysin and leave about one-half of any β-haemolytic activity intact (Llewellyn Smith & Price, 1938*a*). Serial dilutions commencing at 1/5 of each of the supernatants in 20 % broth saline were made in duplicate rows of tubes. Equal volumes of 2 % washed rabbit corpuscles and of 2 % sheep corpuscles were added to each tube of the first and second rows respectively. Readings were made after incubation for 1 hr. in a 37° water-bath and again after leaving in the refrigerator at 4° overnight. The haemolytic titre was recorded as the reciprocal of the highest dilution producing distinct naked eye haemolysis. Table 2 shows the results obtained with the parent strains. None of the variants showed any haemolysin by this method.

(*b*) *Tests with antitoxin in blood agar plates.* Plates of 2 % rabbit and 2 % sheep blood agar were used. A circular well about ¾ in. in diameter was cut out of the centre of the agar and was filled with agar containing a high concentration of the staphylococcus antitoxin. Radial streaks of the staphylococci were made radiating from the edge of the antitoxin well. After incubation at 37° in 20 % CO_2 for 48 hr. the plates were examined for haemolysis. The plates were then left at 4° for 16 hr. and re-examined.

Table 2. *Tube tests for haemolysin on parent strains*

Maximum titre of haemolysis.

	2 % rabbit cells				2 % sheep cells			
	Unheated supernatant		Heated supernatant		Unheated supernatant		Heated supernatant	
Strain	1 hr., 37°	1 hr., 37°; 16 hr., 4°	1 hr., 37°	1 hr., 37°; 16 hr., 4°	1 hr., 37°	1 hr., 37°; 16 hr., 4°	1 hr., 37°	1 hr., 37°; 16 hr., 4°
A	40	160	0	10	10	40	0	0
B	10	20	0	0	0	10	0	0

In both rabbit and sheep blood agar plates strains A and B produced a wide zone of lysis with hazy margins (α-haemolysis). This haemolysis was inhibited by the antitoxin for some distance and a narrower zone of sharply defined lysis was thus disclosed which extended uninhibited right up to the antitoxin well.

The variants produced narrow zones of lysis with sharp edges, that of A VIII being the narrowest. The haemolysis produced by all the variants was not inhibited by the antitoxin.

There was no obvious difference between the lysis observed on sheep and on rabbit blood agar with any strain, nor was there any change after refrigeration.

From the observations on blood plates we conclude that strains A and B produced α-haemolysin and another lysin, not neutralized by our antitoxin, whose characters are those of α_2- or γ-haemolysins (Morgan & Graydon, 1936; Llewellyn Smith & Price, 1938*b*). All the variants had lost the ability to produce α-toxin, although still producing a lysin similar to the second haemolysin produced by the parent strains.

Dermonecrotic effect. Intradermal injection of 0·2 ml. of each supernatant was made into the depilated back of a rabbit. Results were read after 48 hr. Necrosis of the skin was widespread with supernatants from A and B. A 10 showed a marked zone of erythema with possible early necrosis, A III moderate erythema and B 4 only slight erythema. The control fluid produced no change.

Leucocidin. The accepted method is that described by Panton & Valentine (1932). In addition two other methods were devised.

(1) *Panton and Valentine method.* Incubation of washed blood cells with filtrates from the staphylococcal cultures was carried out for 1, 2, 4 and 24 hr. Films were stained with Leishman stain and examined for the presence of

polymorphonuclear (P.M.N.) leucocytes. Using this technique, results were equivocal with the shorter times of incubation. Filtrates from the parent strains A and B after 4 hr. incubation with washed cells caused changes in the P.M.N. leucocytes but these were only indisputable after 24 hr. incubation. Filtrates from the variants showed no leucocidal activity, the appearances being the same as those in which the control fluid had been used. It may be that the filtrates used contained too little leucocidin for its action to be detectable in less than 24 hr. by this method.

(2) *Leucocyte emigration method.* Emigration on the surface of glass slides (Wright, 1918) was used to obtain a concentrated collection of leucocytes. Three paraffin framed cells were made on each of several slides. Three drops of fresh unclotted blood were dropped into each cell and incubated for ½ hr. in a moist chamber to allow emigration of the P.M.N. leucocytes on to the surface of the slide. The clot was then thrown off. The slides, with emigrated leucocytes, were gently washed several times with the fluid to be tested. Three drops of the fluid were then dropped on to the cells which were reincubated in the moist chamber. A set of five slides for each strain and for the control was used. After 1, 2, 3, 4 and 24 hr. one slide from each set was taken out, the fluid was thrown off and the slide was allowed to dry. The cells were stained with Leishman stain and examined microscopically.

The leucocytes on the slides which had been in contact with supernatants from the control and from the variants at all times showed no differences. The supernatant from strain A and, to a lesser extent, from strain B caused progressive damage to the P.M.N. leucocytes which could readily be followed. After incubation for 1 hr. the nuclei were swollen and seemed less dense and clearly defined than normal. After 2, 3 and 4 hr. the damage was very advanced, the leucocytes were swollen and their nuclear material appeared to be scattered all over the cells. After 24 hr. most cells were destroyed leaving only debris and 'ghost cells' (Pl. 1, fig. 2).

(3) *Slide cell method.* The preceding methods depend on direct observation of morphological changes occurring in the P.M.N. leucocytes. It has been shown that the bactericidal power of the blood is destroyed by removal or destruction of leucocytes. Hence we thought that leucocidin might be detected by an effect on the bactericidal power of blood. For this slide cells were used with enterococcus as the test organism, since this is readily destroyed by normal P.M.N. leucocytes, and, since the organism used was haemolytic, growth was easy to observe. Blood from a normal subject was centrifuged, the serum removed and kept in a sterile tube. The deposited blood cells were washed three times in physiological saline. Equal volumes of sterile staphylococcal filtrate and washed blood cells were mixed in tubes and incubated at 37° for 2½ hr. with periodic shaking to keep the contents mixed. At the end of this time 25 cu.mm. of this mixture, 25 cu.mm. of the sterile serum and 5 cu.mm. of a 1:1000 dilution of an overnight broth culture of enterococci were mixed in a sterile pipette. These mixtures were introduced into slide cells, which were then sealed. After incubation at 37° overnight the slide cells were examined and the number of colonies developing observed.

The results (Pl. 1, fig. 3) show that maximum growth occurred in mixtures containing filtrates from A and B. Filtrates from A10 were slightly active, from B4 very slightly active, from AIII inactive. Hence filtrates from the parent strains contained a factor which destroyed the bactericidal power of human blood, whereas the variants produced this factor in small amounts only or not at all.

In order to compare the sensitivity of this method with that of direct methods for measuring leucocidin, filtrate from strain A was used at various dilutions. Pl. 1, fig. 4 shows that the bactericidal power of blood was markedly reduced at a dilution of 1/4 but not at 1/8. Using the same filtrate Panton & Valentine's method and the emigration method showed definite destruction of a large number of P.M.N. leucocytes also at a dilution of 1/4 but not at 1/8.

DISCUSSION

With all the tests used, differences between the parent strains and their avirulent variants were observed which were consistent with the loss of virulence. The number of strains used is too small to draw general conclusions. However, our comparisons between avirulent variants and their virulent parents further supports the generally accepted belief that α-haemolysin, dermonecrotic factor and leucocidin are closely related to virulence.

Panton & Valentine's method of testing for leucocidin was found to be simple and gave definite results especially after 24 hr. incubation. With the weak leucocidin filtrates we were using the P.M.N. emigration method gave quicker results and allowed the demonstration of progressive destruction of the leucocytes.

The ability of staphylococcal filtrates to destroy or diminish the bactericidal power of washed human blood cells, if shown by later work to be due to leucocidin, may allow use of the slide cell method for its estimation. If this is the case, this method may be the one of choice because of its objectivity which is independent of individual interpretation of what constitutes significant leucocyte destruction.

We wish to thank Prof. Sir Alexander Fleming for his helpful encouragement and advice.

REFERENCES

ELEK, S. D. (1948). The recognition of toxicogenic bacteria strains *in vitro*. *Brit. med. J.* i, 493.

MORGAN, F. G. & GRAYDON, J. J. (1936). Toxins of the Staphylococcus with special reference to the estimation of potency. *J. Path. Bact.* 43, 385.

PANTON, P. N. & VALENTINE, F. C. O. (1932). Staphylococcal Toxin. *Lancet*, i, 506.

SMITH, M. LLEWELLYN & PRICE, S. A. (1938*a*). Staphylococcus β Haemolysin. *J. Path. Bact.* 47, 361.

SMITH, M. LLEWELLYN & PRICE, S. A. (1938*b*). Staphylococcus γ Haemolysin. *J. Path. Bact.* 47, 379.

VOUREKA, A. (1952). Induced variations in a penicillin resistant staphylococcus. *J. gen. Microbiol.* 6, 352.

WRIGHT, A. E. (1918). New methods for the study of emigration and of the bactericidal effects exerted in the wound by leucocytes. *Lancet*, i, 129.

WRIGHT, A. E. (1923). New principles in therapeutic inoculation. *Lancet*, i, 365, 417, 473.

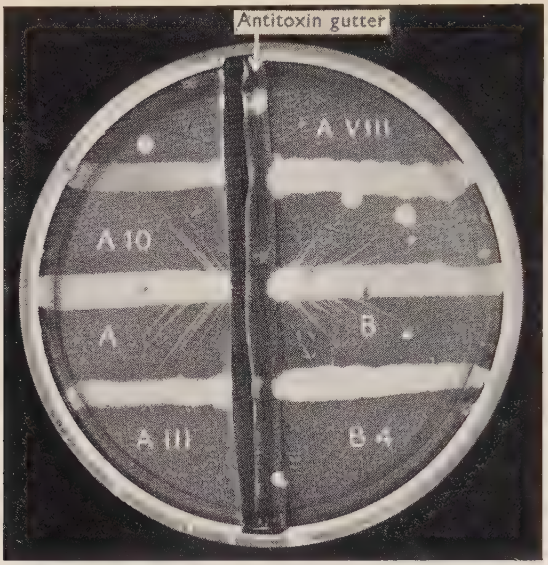
Antitoxin gutter
A VIII
A 10
A
B
A III
B 4

Fig. 1

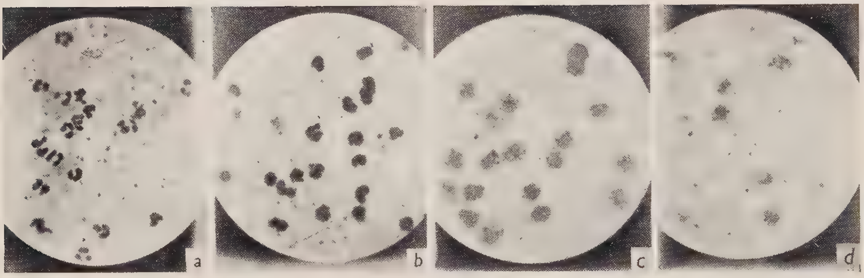

Fig. 2

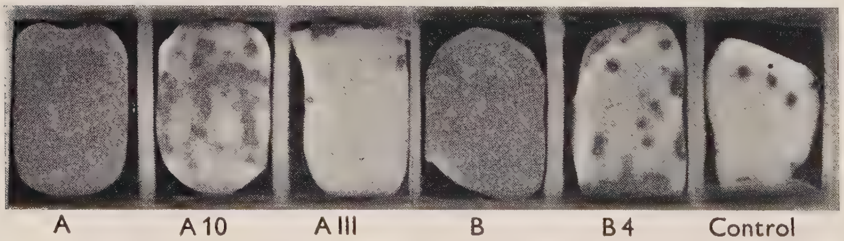
A
A 10
A III
B
B 4
Control

Fig. 3

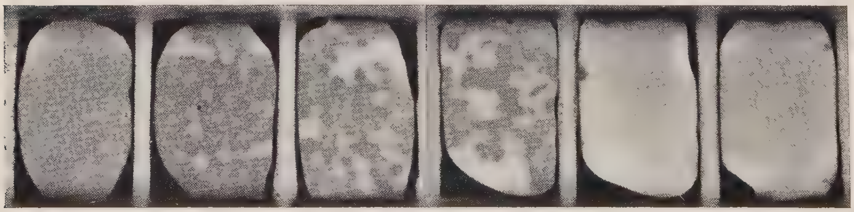

Fig. 4

AMALIA VOUREKA & A. C. OGILVIE—TOXIN PRODUCTION IN STAPHYLOCOCCAL VARIANTS. PLATE 1

EXPLANATION OF PLATE

Fig. 1. Toxin-antitoxin precipitation lines in agar plate with staphylococcal strains streaked at right angles to antitoxin gutter.

Fig. 2. Effect of filtrate from strain A for various times on P.M.N. leucocytes emigrated on to surface of glass slide. *a*, after ½ hr.; *b*, after 2½ hr.; *c*, after 5 hr. and *d*, after 24 hr. incubation.

Fig. 3. Slide cell method. Effect on bactericidal power of washed blood cells after contact with the staphylococcal filtrates (A–B4) using a standard inoculum of enterococcus as indicator organism.

Fig. 4. Slide cell method. Titration of filtrate from strain A. *a* to *e*, dilutions 1/1, 1/2, 1/3, 1/4, 1/8; *f*, control.

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Chamberlain, N., Cutts, N. S. & Rainbow, C. (1952). *J. gen. Microbiol.* 7, 54–60

The Formation of Pigment and Arylamine by Yeasts

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SUMMARY: The growth of several adenine-independent strains of yeasts in defined media containing suboptimal concentrations of biotin and relatively high concentrations of methionine is associated with the formation of a pink pigment in the cells and of an arylamine in the culture fluid. Under similar conditions, pigment and arylamine are not produced by an adenine-requiring strain of *Schizosaccharomyces octosporus*. Culture filtrates containing arylamine promote the growth of *Sch. octosporus* in adenine-free medium, but an adenine-requiring lactobacillus responds only after a considerable lag. The results are interpreted to mean that the arylamine is, or is derived from, a precursor of adenine, whose conversion to adenine is impaired by deficiency of biotin.

This study arose from the observation that the cells of a strain of *Saccharomyces cerevisiae* became pink in a certain nutritional environment. The phenomenon, reported in brief by Cutts & Rainbow (1950*a*) and in greater detail below, may throw light on the biosynthesis of adenine and on the role of biotin in the cell.

METHODS

Experimental cultures. Tube cultures (6 ml.) were carried out as described by Northam & Norris (1951). Cultures of 100 ml. were grown in 250 ml. Erlenmeyer flasks. Incubations were at 25°.

Test organisms and inocula. For most of this work, Yeast 47 (Rainbow, 1948) was used. This yeast requires exogenous *p*-aminobenzoic acid (*p*-AB), but will grow in its absence when provided with relatively high concentrations of methionine, adenine and histidine (Cutts & Rainbow, 1950*b*). Other organisms are listed in the appropriate experimental section. The strain of *Schizosaccharomyces octosporus* was that employed by Northam & Norris (1951). The organisms were maintained, and the inocula for tube tests prepared, as described by Cutts & Rainbow (1950*b*). Proportionately larger inocula were used for the larger test cultures.

The heterofermentative strain of lactobacillus used was maintained in liquid culture in clarified 10 % malt extract supplemented with 5 % of yeast autolysate. This organism, for which we are indebted to Mr W. B. Moore, was isolated from a brewer's pitching yeast and requires an exogenous source of adenine.

Media. The basal medium was that described by Cutts & Rainbow (1950*b*) except that biotin was omitted, 0·10 μg. *p*-AB/ml. added, and the pH adjusted to 4·8. For tests involving *Sch. octosporus*, the basal medium was supplemented with 100 μg./ml. each of DL-methionine and L-histidine HCl.

The medium used to test the growth response of the lactobacillus was a modification of that used for the microbiological assay of nicotinic acid (Analytical Methods Committee, 1946) but from which adenine was omitted.

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Measurement of growth. The turbidities of 6 ml. tube cultures were measured *in situ* in the Hilger 'Spekker' absorptiometer (Northam & Norris, 1951), using distilled water as reference liquid. For determinations on larger cultures, samples of homogeneous yeast suspension were withdrawn and read in the 1 cm. Spekker cell. Weight of cell dry matter (Fig. 2) was determined from turbidity readings by use of a calibration curve.

Determination of arylamine. Culture filtrates were obtained by centrifuging 6 ml. cultures (or 6 ml. samples of larger cultures). The supernatant was poured off as completely as possible, and arylamine in the filtrate determined by the diazotization and coupling reaction of Bratton & Marshall (1939). To determine 'non-acetylable' arylamine, the filtrate was treated with one-tenth of a volume of acetic anhydride at room temperature for ½–1 hr. before diazotization and coupling. Concentration of arylamine was expressed as the absorption of the azo colour in a 1 cm. cell, measured in the Spekker absorptiometer using Ilford filter no. 603 (peak transmission 5000 A.). The absorption spectra of the azo compounds were determined with the same instrument using a range of filters. Since each filter transmits over a range of wavelengths, such spectra provide only a generalized outline, but which is useful for purposes of differentiating between (qualitatively) different azo compounds.

RESULTS

Pigment and arylamine formation by Yeast 47

Cultures of Yeast 47 were grown in tubes of basal media, each containing suboptimal concentrations of inositol, pantothenate, biotin and *p*-AB, and the effect of added methionine was tested in each case. Pigment was formed only in cultures which were both relatively deficient in biotin and which contained added methionine. Pigmentation of the cells was appreciable in the presence of 50μg. DL-methionine/ml., but more marked with 500μg./ml.

Culture filtrates from pink cells, but not those from normal white cells, gave an arylamine reaction much too intense to be ascribed to the *p*-AB content of the medium. The reactive compound differed from *p*-AB with respect to the absorption spectrum of the azo colour (Fig. 1) and in that it was non-acetylable.

Tests in which L- and D-methionine were substituted for the DL-compound showed that the substance active in promoting pigment and arylamine formation was the L-methionine, the D-isomer being inactive.

Other conditions being suitable, cultures were substantially free from pigment and arylamine when any of the following four sets of conditions were observed: (*a*) the concentration of D-biotin was raised to 0·08 mμg./ml. (Fig. 2); (*b*) *p*-AB was omitted from the medium and replaced by 100μg./ml. each of adenine and L-histidine HCl (Table 1); (*c*) 100μg. adenine/ml. was included in the basal medium (Table 1); (*d*) DL-methionine was absent or replaced by an equal concentration of any of the following amino-acids: DL-alanine, DL-α-aminobutyric acid, L-arginine, L-aspartic acid, L-cysteine, L-cystine, L-histidine, DL-homocystine, L-hydroxyproline, L-glutamic acid, glycine, L-leucine, L-lysine, DL-norleucine, DL-norvaline, DL-phenylalanine, L-proline, DL-serine, DL-threonine, L-tyrosine, DL-valine. DL-Tryptophan also failed to induce pigment formation, but in this case arylamine was not tested for, since the amino-acid itself gave a coloured compound on diazotization and coupling.

Table 1. *Arylamine formation by Yeast 47 in the presence and absence of adenine*

Six ml. cultures in basal medium containing 0·02 mμg. D-biotin/ml. Series A contained 10 mμg. *p*-AB/ml.; series B contained no *p*-AB, but 100μg./ml. each of adenine and L-histidine HCl; series C contained 10 mμg. *p*-AB and 100μg. adenine/ml. Incubation time 90 hr.

	DL-methionine (μg./ml.)	Growth (Spekker reading)	Colour of cells	Non-acetylable arylamine (absorption units)*
Series A	0	0·53	White	0·052
	125	0·52	Pink	0·227
	250	0·50	Pink	0·281
	500	0·48	Pink	0·279
Series B	0	0·32	White	0·018
	125	0·49	White	0·018
	250	0·50	White	0·019
	500	0·52	White	0·018
Series C	0	0·44	White	0·019
	125	0·44	White	0·031
	250	0·45	Sl. pink	0·070
	500	0·44	Sl. pink	0·080

* Absorption units = concentration of arylamine expressed as the absorption of the azo colour in a 1 cm. cell, measured in Spekker absorptiometer using Ilford filter no. 603 (peak transmission 5000 A.).

Fig. 2, in which the ratio arylamine reading/dry weight of cells is plotted against concentration of biotin, shows that arylamine formation per unit weight of cell substance increased as biotin deficiency became more marked and as methionine concentration increased. Maximum arylamine formation occurred in cultures containing approximately 500μg. DL-methionine and 0·02 mμg. D-biotin/ml. The maximum was reached in 96 hr., arylamine concentration diminishing thereafter with time (Table 2).

Table 2. *Progress of arylamine formation by Yeast* 47

Cultures in basal medium containing 0·01 mμg. D-biotin and 500μg. DL-methionine/ml.

Incubation (days)	Growth (Spekker reading)	Arylamine absorption units*
2	0·14	0·065
3	0·21	0·173
4	0·25	0·240
5	0·26	0·218
6	0·30	0·178
9	0·36	0·105
11	0·39	0·083

* See footnote to Table 1.

Arylamine and pigment formation by other yeasts

The following strains of biotin-dependent yeasts and yeast-like organisms, maintained in this laboratory, produced pigment and arylamine when grown for periods up to 150 hr. in defined medium partially deficient in biotin and containing 500 μg. DL-methionine/ml. namely: *S. carlsbergensis* 4228, five single-cell isolates of brewer's top fermentation yeasts (*S. cerevisiae*) and a strain of *Brettanomyces*. Pigment was also formed by *Saccharomycodes ludwigii* and *Sch. pombe*, but these culture filtrates gave a relatively feeble reaction for arylamine. *Kloeckera brevis* and *K. apiculata* formed little and no pigment respectively and little arylamine.

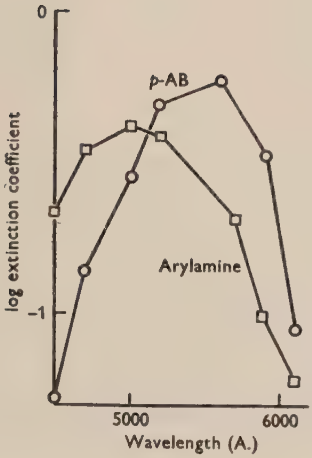


Fig. 1

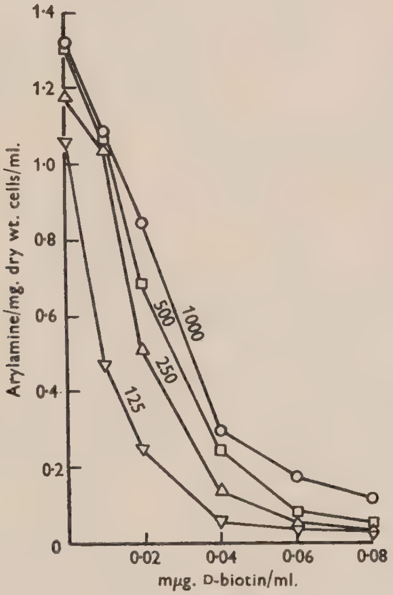


Fig. 2

Fig. 1. Absorption spectra of diazotized and coupled *p*-AB and arylamine.

Fig. 2. Relationship of arylamine formation by Yeast 47 to concentration of biotin and methionine. The figures above the curves are concentrations of DL-methionine in μg./ml.

Neither pigment nor arylamine was produced in cultures of the adenine-dependent strain of *Sch. octosporus* (Northam & Norris, 1951).

Growth-promoting properties of culture filtrates containing arylamine

Neither *Sch. octosporus*, which requires exogenous adenine, nor Yeast 47 when grown in media from which *p*-AB is omitted (so that it is unable to synthesize adenine) forms appreciable arylamine. This suggests that when arylamine accumulates in cultures independent of exogenous adenine, it may do so as a result of a block in adenine synthesis. Arylamine, or a substance from which arylamine is derived, may be therefore a precursor of purine.

Culture filtrates containing arylamine were therefore tested for ability to replace adenine in the nutrition of *Sch. octosporus* and the heterofermentative lactobacillus, both of which require exogenous adenine for growth.

Cultures (100 ml.) of Yeast 47 were grown in basal medium supplemented (1 ml.) as follows: medium I, 2 mμg. D-biotin; medium II, 0·01 mμg. D-biotin; medium III, 0·01 mμg. D-biotin and 500 μg. DL-methionine. After 96 hr., the

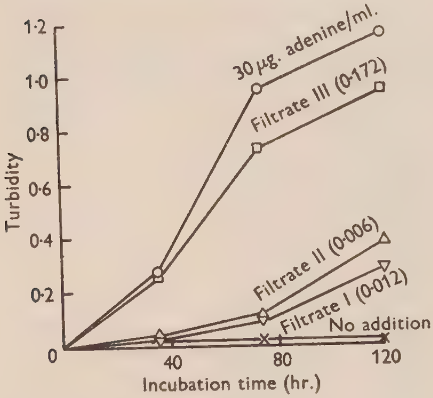


Fig. 3

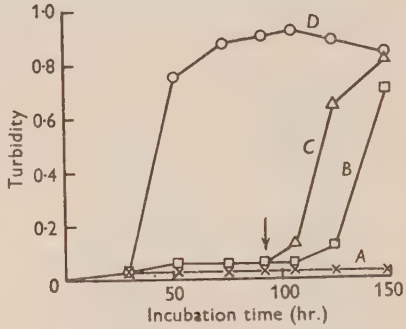


Fig. 4

Fig. 3. Response of *Sch. octosporus* in adenine-free medium to culture filtrates containing arylamine. Filtrates were added at the rate of 0·3 ml. (expressed as original filtrate)/ml. of medium. Arylamine contents, in terms of the original filtrates, are in brackets above the appropriate curves.

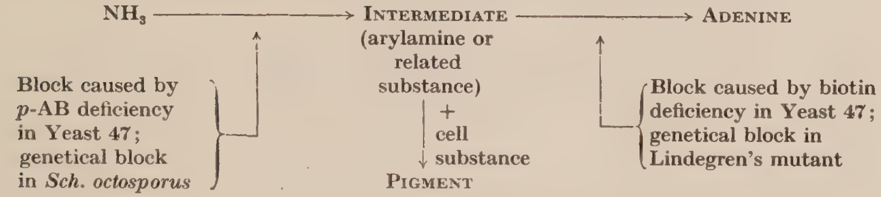
Fig. 4. Response of an adenine-requiring lactobacillus in adenine-free medium to a culture filtrate containing arylamine. Curve *A*, no added filtrate; curve *B*, +1 ml. filtrate/ml.; curve *C*, as *B*, but 10 μg. adenine/ml. added (↓) after 91 hr.; curve *D*, +10 μg. adenine/ml. The filtrate was identical with filtrate III (Fig. 3).

cultures were centrifuged, the supernatant fluids filtered through Whatman no. 44 paper and the filtrates concentrated *in vacuo* below 40° to one-sixth volume. After withdrawing a sample for arylamine determination the concentrates were tested for growth-promoting activity for *Sch. octosporus* and the lactobacillus in adenine-free media. Culture filtrate III (which contained arylamine) was much superior to filtrates I and II (which contained little or no arylamine) as a substituent for adenine in the nutrition of *Sch. octosporus* (Fig. 3). For the lactobacillus, filtrate III replaced adenine after a considerable delay (148 hr.), whereas the response to added adenine was almost immediate (Fig. 4). This delay was not caused by inhibitors added with the filtrate, since addition of adenine after 91 hr. evoked an immediate response. The delayed response of the lactobacillus to filtrate III thus indicates that the active substance in the filtrate is not adenine itself.

DISCUSSION

In this work, the formation of pigment by cells was invariably associated with the presence of arylamine in the culture filtrate. However, pigment and arylamine are not identical, since filtrates containing relatively high concentrations of the latter are usually (but not invariably) colourless. Pigment may result from the interaction of the arylamine with some cell constituent.

Our pigment may be identical with, or related to, that reported by Lindegren & Lindegren (1947) who associated the formation of a pink pigment by certain mutants of *S. cervisiae* with a block in adenine synthesis, coupled with the presence of excess methionine and with other substances. Assuming such a relationship, the following scheme might represent the data:


NH_3
INTERMEDIATE
(arylamine or related substance)
+ cell substance
PIGMENT
ADENINE
Block caused by *p*-AB deficiency in Yeast 47; genetical block in *Sch. octosporus*
Block caused by biotin deficiency in Yeast 47; genetical block in Lindegren's mutant

The Lindegrens suggested that the accumulation of an intermediate by their mutants caused pigment formation. Provided there is no further synthetic disability between the intermediate and adenine, the former (or an arylamine derived from it) might be expected to replace adenine in the nutrition of adenine-dependent micro-organisms, such as we have demonstrated. That arylamine did not accumulate in cultures containing more nearly optimal quantities of biotin suggests that, in Yeast 47, biotin may function in converting the intermediate to adenine.

Stetten & Fox (1945) observed the accumulation of diazotizable amine in cultures of *Escherichia coli* under conditions of sulphonamide bacteriostasis. This amine, 5(4)-amino-4(5)-imidazolecarboxamide, was regarded by Shive, Ackermann, Gordon, Getzendaner & Eakin (1947) as an intermediate in purine biosynthesis and may be related to our amine, although the latter appears distinct with respect to the absorption maximum of the azo compound and in being relatively insensitive to oxidation by a current of air passed through solutions overnight.

That pigment and arylamine formation took place in other species and genera of biotin-dependent yeasts suggests that these phenomena reflect some abnormality of fundamental metabolic processes common to several yeasts and yeast-like organisms.

REFERENCES

Analytical Methods Committee (1946). Report on the microbiological assay of riboflavin and nicotinic acid. *Analyst*, 71, 397.

Bratton, A. C. & Marshall, E. K. (1939). A new coupling component for sulphanilamide determination. *J. biol. Chem.* 128, 537.

Cutts, N. S. & Rainbow, C. (1950*a*). Methionine and the formation of pigment by yeasts. *Nature, Lond.* 166, 1117.

Cutts, N. S. & Rainbow, C. (1950*b*). Studies of a yeast exacting towards *p*-aminobenzoic acid. *J. gen. Microbiol.* 4, 150.

Lindegren, C. C. & Lindegren, G. (1947). Depletion mutation in *Saccharomyces*. *Proc. nat. Acad. Sci., Wash.* 33, 314.

Northam, B. E. & Norris, F. W. (1951). Growth requirements of *Schizosaccharomyces octosporus*, a yeast exacting towards adenine. *J. gen. Microbiol.* **5**, 502.

Rainbow, C. (1948). Para-aminobenzoic acid a growth factor for certain brewer's yeasts. *Nature, Lond.* **162**, 572.

Shive, W., Ackermann, W. W., Gordon, M., Getzendaner, M. E. & Eakin, R. E. (1947). 5(4)-Amino-4(5)-imidazolecarboxamide, a precursor of purines. *J. Amer. chem. Soc.* **69**, 725.

Stetten, M. R. & Fox, C. L. (1945). An amine formed by bacteria during sulphonamide bacteriostasis. *J. biol. Chem.* **161**, 333.

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Pereira, H. G. & Travassos, J. (1952). *J. gen. Microbiol.* 7, 61–68

A Quantitative Agglutination Method for the Study of the Specific Agglutination of Micro-organisms

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SUMMARY: A quantitative method for the study of the specific agglutination of micro-organisms is described. The degree of agglutination is estimated from the sedimentation curves of suspensions to which varying amounts of serum are added, which are compared with that of a control suspension without serum. The time of centrifugation required for sedimentation of 50 % of the organisms in the serum-suspension mixtures related to the corresponding time for the control suspension gives the relative 50 % sedimentation times which, within a certain region, show a linear relation with the amounts of serum used. The activity of sera is expressed by their agglutinin content and by their agglutinating capacity, given by the slope of the time-serum regression line.

Rickettsias of the typhus group and their corresponding sera are clearly distinguished by the method described. Proteus OX19 agglutinating sera of different origins (pregnant women, murine typhus and Proteus-immune rabbit), with the same agglutinating titre by the dilution method, are distinguished according to their origin by the quantitative method. The application of the method to the study of other groups of micro-organisms is suggested.

The study of the specific agglutination of bacteria, rickettsias and viruses by immune sera is usually made by the serial dilution method, and the results are expressed as the highest dilution of a serum which causes a standard degree of agglutination. To obtain greater uniformity and reproducibility of the results of such tests, Hedén (1946) described a method for the determination of the 50 % end-point based on the method of Reed & Muench (1938) for the evaluation of end-points in animal tests. This method has the advantage of taking into consideration a number of dilutions for the evaluation of the titre but it suffers from the influence of the personal bias which may be considerable when agglutination tests are read by different workers. The method described by Hirst & Pickles (1942) for the study of virus haemagglutination, if applied to the study of the agglutination of micro-organisms, as suggested by Kabat & Mayer (1948), would probably yield valuable results. In this test, the degree of agglutination in individual tubes is estimated by measuring the density of the layer of unsedimented cells after a fixed period of time, and the course of the haemagglutination is translated by a sigmoid curve. In our experience (unpublished data) a similar type of curve was observed for bacterial and rickettsial agglutination. A great advance in the study of the agglutination reaction was made with the introduction of the chemical quantitative method of Heidelberger & Kabat (1934*a*, *b*) in which the amount of antibody combined with agglutinated bacteria is measured by weight. This method, making possible the study of the course of bacterial agglutination as a function of antigen and antibody, is a valuable tool for research, but its practical applications are limited by the technical difficulties involved.

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In the present paper we describe a method in which the degree of agglutination is estimated by the sedimentation curves of suspensions to which various amounts of serum are added; these are compared with the sedimentation curve of a control suspension without serum. The sedimentation curves are built by reading the light extinction of suspensions in a photoelectric colorimeter after successive periods of centrifugation. The term sedimentation is used in a wide sense, as the variations of the light extinction depend both on the sedimentation proper and on the aggregation of the organisms. In such tests, a linear relationship is observed, within a certain range, between the amount of antibody present and the relative time necessary for 50 % of the bacteria or rickettsias to be deposited.

MATERIAL AND METHODS

Antigens

Rickettsial suspensions were prepared from infected chick embryo yolk-sacs, purified by ether treatment and differential centrifugation. Murine (Wilmington strain) and classical (Breinl strain) typhus suspensions were prepared by method 2 of Topping & Shepard (1946). The suspension of Q fever rickettsias was prepared by method 1 of the same authors. The suspensions were washed shortly before use by two centrifugations in an angle-head centrifuge at 4000 r.p.m. for 1 hr. and re-suspended in 0·2 % formalized 0·005 M phosphate-buffered (pH 7·3) saline (Kent, Bukants & Rein, 1946), the diluent used throughout. The density of the suspensions was adjusted so as to give, when diluted 1/10, a light extinction reading of 50.

Bacterial suspension. Ethanol-treated suspension of Proteus OX 19 was prepared according to the method of Bridges (1944). The density was adjusted so as to give, when diluted 1/10, a light extinction reading of 100.

Sera

Murine typhus serum. Pooled sera from guinea-pigs bled about 30 days after infection with the Wilmington strain.

Classical typhus serum. Obtained from a guinea-pig infected with a yolk-sac suspension of the Breinl strain and bled 30 days later.

Q fever serum. Guinea-pig immune serum kindly supplied by the late Dr R. R. Parker, from the Rocky Mountain Laboratory, Hamilton, Montana.

Proteus antisera. Obtained from human cases of murine typhus, from pregnant women, and from a rabbit immunized with an ethanolic suspension of Proteus OX 19.

Measurement of opacity

The density of the suspensions was determined by the light extinction observed in a Klett-Summerson photoelectric colorimeter with a 54 filter. A special holder was adapted to take standardized 13 × 100 mm. tubes. The window was slightly reduced to allow the use of 1 ml. volumes. Within the region in which we worked, a linear relationship was obtained between the concentration of the suspension and the light extinction, as seen in Table 1 in which the light extinctions of various concentrations of a Proteus OX 19 suspension are given. The value of 100 % was chosen arbitrarily to give a reading of approximately 100.

Table 1. *Opacity measurements of Proteus OX* 19 *suspensions*

Suspension (ml.)	Saline (ml.)	Suspension (%) (x)	Light extinction (y)
0·1	0·9	10	9
0·2	0·8	20	19·5
0·3	0·7	30	30·5
0·4	0·6	40	41
0·5	0·5	50	52
0·6	0·4	60	60
0·7	0·3	70	70
0·8	0·2	80	81
0·9	0·1	90	92
1·0	—	100	99

The parameters of the line $y = bx + a$ gave values of 0·154 and 0·99 respectively for a and b, allowing the direct reading of the percentage of bacteria in suspension from the light extinction.

For economy, less concentrated rickettsial suspensions were used and the 100 % density was standardized to give a light extinction reading of 50. The same linear relationship was observed as in bacterial suspensions, and the percentages of rickettsias were given by the light extinction readings multiplied by 2.

Agglutination methods

Serial dilution tests

Serial twofold dilutions of the serum were made in saline and an equal volume of the suspension was added. Proteus OX 19 agglutination was done in 12 × 75 mm. tubes with a total volume of 1 ml. and the rickettsial agglutinations in 10 × 75 mm. tubes with a total volume of 0·2 ml. The tests were incubated in a water-bath at 37° for 2 hr. and left overnight at 4°. The titres represent the highest final dilution of serum causing definite agglutination. The 50 % end-points were calculated according to the method described by Hedén (1946). The density of all the suspensions was adjusted to give a light extinction reading of about 85.

Quantitative agglutination test

0·1 ml. volumes of the suspension were distributed with a 0·2 ml. pipette at the bottom of a series of standardized 13 × 100 mm. tubes. Increasing volumes (in arithmetical progression) of an appropriate dilution of serum were added and the volumes in all tubes were made up to 1 ml. with buffered saline. The control tube received 0·1 ml. of the suspension and 0·9 ml. of buffered saline. The tubes were shaken, left to stand for a few minutes, and the light extinctions were read with the photoelectric colorimeter to check the 100 % density. The tubes were then incubated in a water-bath at 37° for 2 hr. and were then centrifuged in a horizontal centrifuge at a speed previously determined to yield 50 % sedimentation within 10 and 20 min. (about 2200 r.p.m. for rickettsial suspensions and 1000 r.p.m. for bacterial suspensions). After each 5 min. period the tubes were carefully removed from the centrifuge and the light extinctions determined with the photoelectric colorimeter. Centrifugation was continued until over 50 % of the organisms in the control tube were deposited; usually three or four periods of 5 min. were sufficient (Table 2). The tubes were then centrifuged at high speed for all the organisms to be deposited, and the light extinctions of the supernatants were read; these values were deducted from the previous readings obtained with the corresponding tubes to eliminate error introduced by haemolysis or opalescence of the sera. The percentage of organisms deposited after each period of centrifugation was calculated and plotted against time. The 50 % sedimentation times ($T50$) were read from the curves (Fig. 1). The $T50$ of the control was adopted as the unit time (U) and the relative 50 % sedimentation times (T) were given by the relation $T50/U$, which may vary from very nearly O (complete agglutination) to 1 (absence of agglutination). The relative 50 % sedimentation times plotted against the amounts of serum (S) gave points approximately on a straight line (Figs. 2–5), and the reaction may be expressed by the equation $T = bS + a$. The agglutination unit was defined as the amount of serum giving a relative 50 % sedimentation time of 0·5, that is, an amount of serum with which the 50 % sedimentation time is half that of the control. This unit was obtained from the line $T = bS + a$; the parameters a and b were calculated by the method described by de Almeida (1951).

Table 2. *Quantitative agglutination test with Proteus OX* 19

Suspension (ml.)	Serum 1/200 (ml.)	Saline (ml.)	Serum concentration (ml.) (S)	Sedimentation 5 min.	Sedimentation 10 min.	Sedimentation 15 min.	T 50*	$T50/U$† (T)
0·1	0·4	0·5	0·0020	27	43	56	12·5	0·86
0·1	0·5	0·4	0·0025	31	48	61	10·5	0·72
0·1	0·6	0·3	0·0030	38	56	70	8·5	0·58
0·1	0·7	0·2	0·0035	41	61	72	6·5	0·44
0·1	—	0·9	—	18	38	52	14·5	—

* Time in minutes for 50 % sedimentation; read from the curves.
† U = T 50 for the control tube.

RESULTS

Agglutination of rickettsias

Q fever immune guinea-pig serum, with a titre of 1/1280 by the dilution method, showed by the quantitative method an agglutination unit of 0·00075 (1333 units/ml.). The relative sedimentation times plotted against the amounts of serum gave points approximately on a straight line with a regression coefficient of −628.

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Murine and classical typhus sera were tested against both the homologous and the heterologous suspensions; the results are shown in Table 3 and in Figs. 3 and 4.

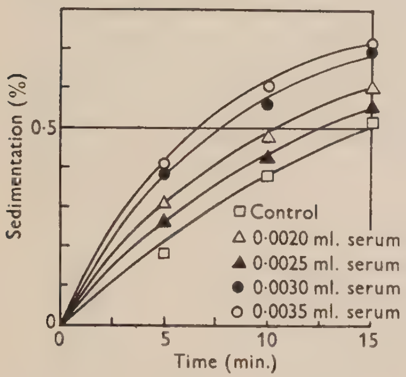

Fig. 1. Sedimentation curves of Proteus OX19 suspension in the presence of varying amounts of agglutinating serum. (From Table 2.)

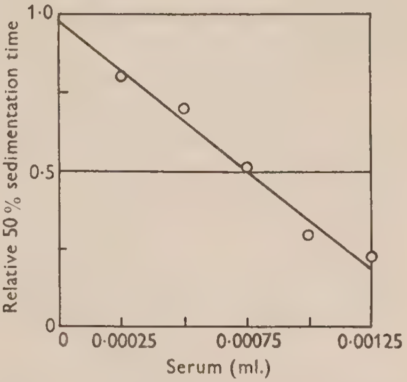

Fig. 2. Agglutination of Q fever rickettsias of homologous serum.

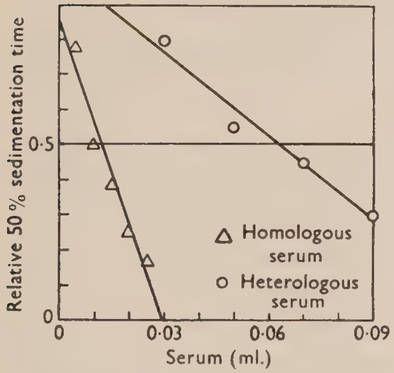

Fig. 3. Agglutination of murine typhus rickettsias by murine and classical typhus sera.

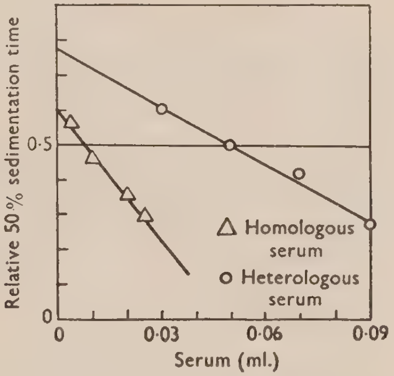

Fig. 4. Agglutination of classical typhus rickettsias by classical and murine typhus sera.

Table 3. *Agglutination of murine and classical rickettsias by homologous and heterologous sera*

			Quantitative method		
Serum	Antigen	Titre by dilution method	Agglutination unit	Units/ml.	Regression coefficient
Murine	Murine	1/80	0·012	83·3	−29·4
Classical	Murine	1/40	0·0625	16·0	− 8·0
Murine	Classical	1/20	0·05	20·0	− 5·2
Classical	Classical	1/80	0·0085	117·6	−12·5

It was possible to differentiate the two systems by both the quantitative and the serial dilution methods. The former method allowed a clear differentiation by two characteristics: the agglutination unit, which was always lower for the homologous than for the heterologous systems; and the regression coefficient, with higher values for the homologous than for the heterologous systems.

In the serial dilution method, both sera gave titres of 1/80 with the homologous antigens and lower titres with the heterologous antigens; the differences however, were of the order of one or two dilutions, and, in view of the difficulty in reading the finer degrees of agglutination, the end-point was not always easy to define. We found the estimation of the 50 % end-point (Hedén, 1946) to be impractical in rickettsial agglutination tests because of the difficulty in distinguishing five or even four different grades of agglutination.

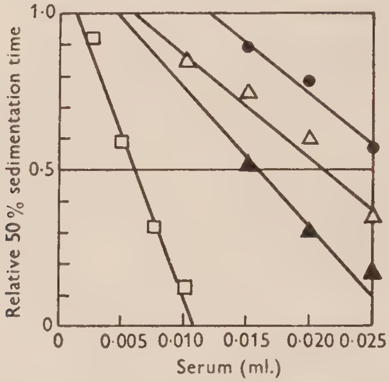

Fig. 5. Agglutination of Proteus OX19 by sera of different origins. □, rabbit Proteus antiserum; ▲, murine typhus serum; △, pregnancy serum A; ●, pregnancy serum B.

Agglutination of Proteus OX 19 (*Weil-Felix test*)

In Table 4 we give the results of the serial dilution and of the quantitative agglutination methods obtained with four sera, all of which gave a titre of 1/320 by the first method.

Table 4. *Agglutination of Proteus OX* 19 *by sera of different origins*

	Dilution method		Quantitative method		
Sera	Titre	50 % end-point	Agglutination unit	Units/ml.	Regression coefficient
Rabbit Proteus antiserum*	1/320	1/201	0·006	166	−107
Murine typhus	1/320	1/226	0·016	62	− 44·4
Pregnancy A	1/320	1/201	0·021	47	− 33
Pregnancy B	1/320	1/127	0·027	37	− 32

* 1/32 dilution of a rabbit Proteus antiserum with an original agglutination titre of 1/10,240.

The quantitative method clearly distinguished these sera both by the agglutination unit and by the regression coefficient, the latter providing a particularly clear distinction (Fig. 5). A certain correspondence was observed between the values obtained for the 50 % end-points calculated according to Hedén (1946) and those obtained by the quantitative method, except in the case of the rabbit immune serum in which the 50 % end-point was low in relation to the agglutination unit and to the slope.

DISCUSSION

The linear relationship between the degree of agglutination, judged by the relative 50 % sedimentation time, and the amounts of serum, provides valuable information for the study of the agglutination reaction. The activity of sera studied by this method may be expressed by two characteristics: the agglutinin content, represented by the agglutination unit; and the agglutinating capacity, represented by the slope (b) of the time (T)-serum (S) regression line $T = bS + a$.

In this respect, our results are analogous to those obtained with the quantitative complement-fixation test, in which the complement-fixing capacity of certain systems is better expressed by the slope of the complement-antigen and complement-serum regression lines (Rice, 1946; Wolfe & Kornfeld, 1949).

From our results with Proteus OX 19 agglutination, it is interesting to note that in the case of sera of the same origin (pregnancy) the time-serum regression lines show a striking parallelism (Fig. 5), whereas a comparison of the sera of different origins reveals an increasing slope corresponding to increasing specificity of the reaction. These results are in agreement with the view that the Weil-Felix reaction is due to the presence, in the sera of typhus patients, of an antibody reacting with one of the antigenic groupings of Proteus OX 19 (Weil & Felix, 1921; White, 1933; Castaneda, 1935). They also agree with the observation that the agglutination of Proteus OX 19 by sera from pregnant women is due to a factor distinct from that in sera from typhus patients, as shown by indirect evidence by Travassos, de Gois, Pereira & Bruno-Lobo (1951).

From the results obtained, it may be expected that the application of the present method to the study of other groups of micro-organisms will be of value in providing evidence of inter-group antigenic differences. The method may, with the help of analytical centrifuges, also prove to be of use in the study of the antigenic structure of the smaller viruses. Its use as a routine serological test requires further investigation.

In the case of a few sera tested against rickettsial suspensions, a time-serum linear relationship was not observed. The same sera showed in serial dilution tests a wide range of dilutions with partial agglutination. The significance of these results was not investigated.

We wish to express our thanks to Dr Horacio Cintra for his valuable help and criticism in the course of this work.

REFERENCES

Almeida, M. O. de (1951). Sur la calcul des paramètres des equations de la forme $y=ax+b$. *Ann. Acad. bras. de Ciencias*, **23**, 421.

Bridges, R. F. (1944). Notes on the preparation of suspensions for the Weil-Felix test. *Trans. R. Soc. trop. Med. Hyg.* **37**, 343.

Castaneda, M. R. (1935). The antigenic relationship between *Bacillus proteus* X19 and Rickettsiae. III. A study of the antigenic composition of extracts of *Bacillus proteus* X19. *J. exp. Med.* **62**, 289.

Heden, C.-G. (1946). On the estimation of fifty per cent end-points in serological titrimetry. *J. Path. Bact.* **58**, 477.

Heidelberger, M. & Kabat, E. A. (1934*a*). Chemical studies in bacterial agglutination. I. A micro-method for the quantitative estimation of agglutinins. *Proc. Soc. exp. Biol., N.Y.* **31**, 595.

Heidelberger, M. & Kabat, E. A. (1934*b*). Chemical studies on bacterial agglutination. I. A method. *J. exp. Med.* **60**, 643.

Hirst, G. K. & Pickles, E. G. (1942). A method for the titration of influenza haemagglutinins and influenza antibodies with the aid of a photoelectric densitometer. *J. Immunol.* **45**, 273.

Kabat, E. A. & Mayer, M. M. (1948). *Experimental Immuno-chemistry.* Springfield, Illinois: Charles C. Thomas.

Kent, J. F., Bukants, S. C. & Rein, C. R. (1946). Studies in complement-fixation. I. Spectrophotometric titration of complement; construction of graphs for direct determination of the 50 per cent hemolytic unit. *J. Immunol.* **53**, 37.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty per cent end-points. *Amer. J. Hyg.* **27**, 493.

Rice, C. E. (1946). Studies of the complement-fixation reaction in virus systems. I. Activities of vaccinia virus antigens and antisera. *J. Immunol.* **53**, 225.

Topping, N. H. & Shepard, C. C. (1946). The preparation of antigens from yolk sacs infected with Rickettsiae. *Publ. Hlth Rep., Wash.* **61**, 701.

Travassos, J., de Gois, P., Pereira, H. G. & Bruno-Lobo, M. (1951). Comportamento immunologico de soros de gravidas de cance-nosos sobre O Proteus OX19 e Rickettsiae. *Ann. Microbiol., Rio de Janeiro*, **1**, 21.

Weil, E. & Felix, A. (1921). Über die Beziehungen der Fleckfieberagglutination zum Fleckfiebererreger. *Z. ImmunForsch.* **31**, 457.

White, P. B. (1933). The O-receptor complex of *B. proteus* X19. *Brit. J. exp. Path.* **14**, 145.

Wolfe, D. M. & Kornfeld, L. (1949). The application of a quantitative complement fixation method to a study of Q fever strain differentiation. *J. Immunol.* **61**, 297.

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Ryan, F. J. (1952). *J. gen. Microbiol.* 7, 69–88

Adaptation to use Lactose in *Escherichia Coli*

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SUMMARY: The adaptation of a genetically deficient strain of *Escherichia coli* (*Bacterium coli*) to the utilization of lactose was shown to involve overgrowth of the population by a small number of mutants. The rate of mutation to lactose utilization is about 2×10^{-7} per bacterium per generation. This rate is not influenced by lactose or a series of substances related to that sugar. The distribution of these mutants among different cultures is in agreement with that expected on the basis of random mutation. These results are discussed in connexion with the theories of Hinshelwood on bacterial adaptation, and in relation to the problem of specifically induced mutation.

Bacteria are remarkably plastic in their ability to undergo satisfactory adjustments in new environments. These adjustments are adaptations in the original biological sense of the word whether they are inherited or not, and irrespective of the underlying mechanisms.

The adaptation of a bacterium to grow in one environment may be transmitted to its progeny for a few generations in a different environment, but such an adaptation would be considered to involve a genetic change only if the intracellular factors responsible for the adapted character could be shown to have multiplied in the course of these generations. Thus, in the case of those adaptive enzymes that are simply diluted to the unadapted level during bacterial division in the absence of their peculiar substrates, the adaptation does not involve a genetic change. On the other hand, when the adaptation involves the selective overgrowth of a population by organisms possessing a mutation in a chromosomal or non-chromosomal gene, it may be inherited in another environment because of the multiplication of the mutated factor.

Nevertheless, the criterion of stability alone does not allow a distinction between genetic and non-genetic adaptations because gene mutations are generally reversible. Nor need the distinction be as clear-cut as first indicated. Rather, it is preferable to ask more specific questions about the nature of the adaptive mechanism. First, it is important to know whether an adaptation occurring in a population of bacteria involves all members of the population or whether there is selective overgrowth by a few unusual members. Further, it is necessary to know whether the change has been induced by the new environment or whether it may occur in another, or whether both possibilities exist.

For example, *Escherichia coli* (*Bacterium coli*) strain lac^{+} possessing the inherited ability to grow on lactose is unable to do so at a maximum rate until it has been exposed to the sugar for some time (Monod & Audureau, 1946). This adaptation is due to the increased synthesis of the enzyme lactase in cells exposed to lactose (Cohn & Monod, 1951). Other sugars and glycosides may also induce lactase synthesis, but it appears that the possession of an unsubstituted galactoside radical is an essential but insufficient condition (Monod, Cohen-Bazire & Cohn, 1951). The lactase formed in one cell may be transmitted to its progeny, but since the amount of enzyme increases only in the presence of an inducing substance the adaptation in lactase synthesis is not due to a genetic change.

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It remains a question to what extent genetic factors operate in this sort of adaptation. Lac^- bacteria, from which the lac^+ strain had been obtained by mutation, do not increase their content of lactase in the presence of lactose during a period in which lac^+ bacteria would have become completely adapted. Thus it would seem that the presence of a gene peculiar to lac^+ organisms is necessary for the adaptation discussed above. Yet a culture begun with lac^- bacteria will, after a long lag period, eventually be transformed into one indistinguishable from lac^+. The present paper presents an analysis of the nature of this adaptive response of lac^- bacteria. Two main questions are examined. First, what percentage of the population is involved in the change; and, secondly, is the initial change in this segment independent of the presence of lactose or related compounds? An examination of the first question has already been made for other adaptations (Fildes & Whitaker, 1948; Ryan & Schneider, 1949; Jackson & Hinshelwood, 1949; Barer, 1950) and interpretations have varied for reasons that will become evident. Luria & Delbrück (1943) have developed a method for determining whether the change in the individual bacteria is provoked by the testing substance, but this method has been criticized by Eriksen (1949) and Hinshelwood (1950). In the present paper these criticisms have been taken into account.

MATERIALS AND METHODS

Strain. The organism used in these studies was a strain of *Esch. coli* ML which is able to grow with glucose, maltose, or galactose as sole carbon source in a defined medium devoid of added growth factors. It is, however, unable to use lactose (Monod & Audureau, 1946). This strain is known not to undergo recombination. The mutant strain requiring tryptophan was secured by Monod after ultraviolet irradiation.

The study reported in this paper was concerned with the change from inability to grow on lactose (lac^- bacteria) to the ability to utilize this sugar (lac^+ bacteria). Monod & Cohn (1952) have described a chemical basis for the difference between the two states. When both strains are grown in the absence of lactose they possess only a trace of lactase and are indistinguishable on this basis and in terms of amounts and rates of growth. Upon exposure to lactose, lac^+ bacteria increase their lactase content some hundreds of fold in a few hours while lac^- bacteria are unable to do this, increasing their lactase content little if at all.

Stocks and inocula. Upon receipt all cultures were plated out on a nutrient agar containing beef extract and peptone. Single colonies were picked on to nutrient agar slopes on which also the organisms were subcultured every few months. The characters differentiating one strain from the other were verified initially as was the phage-resistance pattern characteristic of all ML strains used. Inocula were prepared by transferring a small amount of surface growth from an agar slope into 5 ml. of defined medium supplemented with $5 \cdot 6 \times 10^{-3}$ M (0·2 %, w/v) maltose (and 20 μg. DL-tryptophan/ml. when necessary). The test-tubes containing the inocula were shaken in an inclined position at 37° for about 12 hr. after which time there were about 3×10^9 bacteria/ml. in a stationary phase brought about by sugar exhaustion. In the case of tryptophan-requiring bacteria the inocula were washed by two centrifugations and resuspensions in sterile water to remove traces of unused tryptophan.

Defined medium and sugars. The basal medium contained, per litre of water: 13·6 g. KH_2PO_4; 2·0 g. $(NH_4)_2SO_4$; 0·2 g. $MgSO_4 . 7H_2O$; 0·01 g. $CaCl_2$; 0·0005 g. $FeSO_4 . 7H_2O$; and enough (*c.* 8 ml.) 10 N-KOH to bring the solution to pH 7·4. The sugars used were passed through Pyrex UF glass filters for sterilization and were then added (usually in a concentration of 0·2 %, w/v) to the autoclaved medium. One sample of Pfanstiehl β-lactose was recrystallized from water at room temperature three times. The other chemicals used in these experiments have been described by Monod *et al.* (1951).

Defined medium was usually solidified by the addition of 2 % (w/v) washed agar. In this case 4 % agar in water was autoclaved separately and then mixed with an equal volume of basal medium of double strength. In some instances the medium was solidified with 5 or 8 % (w/v) silica gel. The proper amount of Na silicate solution, sterilized by ultraviolet light, was mixed in a Petri dish with sterile basal medium and sugar and neutralized with sterile 2 N-HCl. The bacterial suspension was then mixed and the plate allowed to set, a process that usually took place within 15 min. In some instances the medium and bacteria were added to ultraviolet sterilized silica gel plates prepared and washed free of NaCl by a method kindly demonstrated by Dr Hélène Winogradsky.

Culture conditions. Cultures were always shaken in inclined test tubes at a rate providing maximum aeration at 37°. When necessary, growth was followed by turbidimetric measurements in a Meunier densitometer, by the use of specially constructed T-shaped tubes (Monod *et al.* 1951). With this densitometer changes in density equivalent to about 10^6 bacteria/ml. in the exponential phase of growth can be detected and optical density is directly proportional to bacterial number up to concentrations of about 10^8 bacteria/ml.

Enumeration and characterization of lac^+ *and* lac^- *bacteria*

Total bacterial counts were made of organisms stained with methylene blue in a haemocytometer. Viable counts of liquid cultures were made by plating appropriate dilutions, made in sterile water, into maltose agar (a bottom layer of agar was always present in the plates before the bacterial suspension was introduced). After 36 hr. at 37°, well-formed colonies could be counted. Counts of the number of bacteria in microcolonies, whose diameter had been determined with a micrometer ocular, were made by isolating whole colonies on small blocks of agar by means of the spatulate tip of an inoculation needle under a dissecting microscope, and transferring to 1 ml. lots of sterile water.

After suspension and appropriate dilutions the bacteria from the microcolonies were plated into maltose agar. Independent estimates of the number of bacteria in microcolonies of different diameter were made for all of the conditions tested.

When streaked or plated *on* the surface of eosine methylene blue (EMB) agar or Endo agar lac$^+$ bacteria form blue or red colonies, while the colonies formed by lac$^-$ bacteria are nearly white in colour. After 1 or 2 days the surfaces of lac$^-$ colonies become spotted with coloured papillae that consist of lac$^+$ bacteria (Massini, 1907). Lac$^-$ bacteria plated *in* EMB or Endo agar form colonies which appear light rose after one day, but later they gradually become more intensely coloured. On the other hand, under the same conditions lac$^+$ bacteria form colonies which are deeply coloured at the outset.

By the use of these indicator agars it is possible on a single plate to distinguish a few bacteria of one type in the presence of the other provided that their frequency is not much less than 10^{-3}. Direct plating of about 400 bacteria on the surface of a number of EMB plates showed that in lac$^-$ cultures the frequency of lac$^+$ bacteria was probably $< 7 \times 10^{-5}$, and that lac$^+$ cultures probably contained $< 10^{-4}$ lac$^-$ bacteria. This method is, however, limited by the fact that the bacteria existing as the major component are not prevented from forming good sized colonies. It has not yet been possible to measure smaller frequencies of lac$^-$ bacteria, for attempts to concentrate them by the use of penicillin have failed, for a reason that will become evident (see Results).

It is possible, however, to screen for lac$^+$ bacteria present in very small frequencies in lac$^-$ populations by a special method that takes advantages of the differential ability of the two types to grow on lactose agar. This involves plating a culture whose growth has stopped because of the exhaustion of sugar on to the surface of thick lactose agar plates that have been dried by storage at 37° for 2 days. Between 0·1 and 0·3 ml. bacterial suspension is placed on the surface of the agar and spread with a sterile glass rod until all the liquid has been absorbed and the surface is once again dry. After between 16 and 18 hr. at 37° the lac$^+$ bacteria have formed colonies easily recognizable at a $6{\cdot}3 \times$ magnification under a dissecting microscope. Because of the importance for this analysis of differential viable counts of lac$^-$ and lac$^+$ bacteria in the same culture, the reliability of this method will be examined in some detail under Results.

RESULTS

Growth of lac$^-$ bacteria on lactose

Populations of lac$^-$ bacteria, when serially transferred in defined medium containing maltose, retain their lac$^-$ character. On the other hand, when lac$^-$ bacteria are inoculated into medium containing lactose they form a culture which is lac$^+$ in character. Thenceforth, when such a culture is carried in the absence of lactose for more than 100 generations, it retains that character. To test whether this result was arrived at by a change in all of the bacteria present or by the selection of a few mutants in the inoculum, single lac$^-$ bacteria were inoculated into medium containing $1{\cdot}6 \times 10^{-3}$ M lactose. This inoculation was achieved by introducing an average of 0·90 lac⁻ bacteria grown on maltose into 50 separate 10 ml. cultures. Use of the Poisson formulation allows the calculation that 18 cultures would be expected to have received one bacterium, and 12 cultures more than one. It was found that 26 cultures showed visible turbidity after about 50 hr., demonstrating that single lac⁻ bacteria were eventually able to form populations of bacteria that grow on lactose. The same conclusion may be drawn from the fact that all lac⁻ bacteria spread on the surface of agar containing $1{\cdot}6 \times 10^{-3}$M lactose will form lac⁺ colonies in a matter of days. This would indicate that every lac⁻ bacterium is able to adapt to grow on lactose, were it not for the possibility that the lac⁻ bacteria might first form micropopulations in lactose medium, due to the utilization of other substances present, and subsequently mutate to the lac⁺ condition. In fact, this is what occurs.

Growth of lac⁻ bacteria in the absence of added sugar

Each lac⁻ bacterium grown to sugar exhaustion on maltose, when plated on the surface of lactose agar, will form a circular colony of uniform depth and homogeneous appearance which grows slowly (*c.* 0·06 doublings/hr.) as shown in Fig. 1. The number of cells/colony is directly proportional to the area, indicating that such colonies grow on the surface of agar in a fashion approximating flat cylinders with continually increasing circumferences. When such colonies at any stage of growth are streaked on to an indicator agar they give characteristic lac⁻ reactions. Eventually, however, local thickenings or papillae appear on the surfaces of these colonies, and when this occurs early enough the papillae overgrow the old colony to form a superimposed one that increases in size at a rapid rate (Monod & Audureau, 1946). Inoculation of such papillae, or of superimposed colonies, to indicator agar demonstrates their lac⁺ character. It would appear as though the growth of the lac⁻ microcolony resulted eventually in populations so large as to make mutation to lac⁺ a likely event. The quantitative examination of this possibility will be deferred until later in this paper.

The original growth of the lac⁻ microcolony might be due in small part to the slow utilization of lactose or of impurities not removed by recrystallization. A larger part is due to other carbon sources, since lac⁻ bacteria on agar devoid of lactose form microcolonies almost as large as on lactose agar. When the agar has been carefully washed the microcolonies formed are somewhat smaller. Microcolonies are formed even on silica gels. They grow to a larger size as satellites around lac⁺ colonies, but their presence even in the absence of the latter indicates that the medium forming the base of the gel contains some carbon source.

This possibility was examined by inoculating about 2×10^3 lac⁻ bacteria into 10 ml. liquid medium with and without lactose. From time to time during incubation at 37° samples were taken and plated in Endo agar. The growth curves obtained are shown in Fig. 2. Serial transfer from one culture without lactose to another resulted each time in similar amounts of growth, suggesting that growth was due to a limited amount of carbon source contaminating the medium. In order to locate the source of this contamination water twice distilled over alkaline permanganate was used exclusively, all glassware was carefully cleaned in chromic-sulphuric acid cleaning solution and rinsed in this twice distilled water, care was taken to avoid contact with cotton by the use of tin caps for tubes and flasks, and all media were sterilized by filtration through a Pyrex UF glass filter. Nevertheless, similar results were obtained. Apparently the contaminating carbon source resides in the salts employed. It allows the growth of *c.* 8×10^6 bacteria/ml. and is the equivalent of *c.* 4 μg. sugar/ml. This would involve a contamination of 2 parts/1000 of salt, a possibility that seems not out of the question.

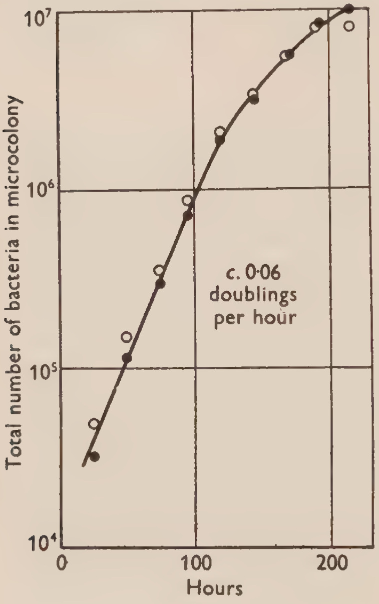

Fig. 1

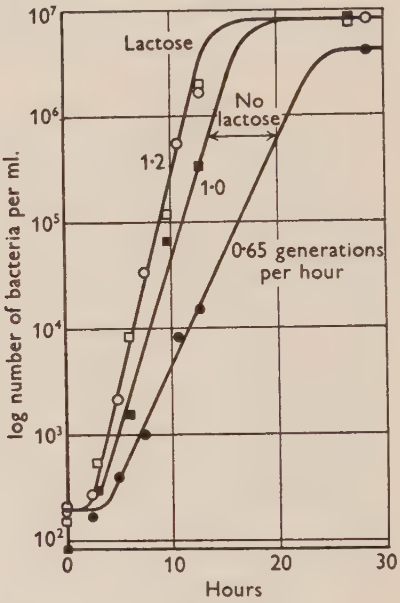

Fig. 2

Fig. 1. The growth of lac⁻ bacteria in a microcolony as determined by measuring the increase in diameter of eight colonies on $1{\cdot}6 \times 10^{-4}$ M lactose agar (—○—○—) and eight colonies on agar to which no carbon source has been added (—●—●—). The colony diameters were converted into numbers of bacteria by the use of the relationship between diameter and bacterial number obtained by plating out microcolonies of different size from both types of agar.

Fig. 2. The growth of lac⁻ bacteria in defined liquid medium with $1{\cdot}6 \times 10^{-3}$ M lactose (—□—□— and —○—○—) and without lactose (—■—■— and —●—●—). The circles represent data obtained when routine procedures were used; the squares, when special precautions were taken to decrease the amount of contaminating carbon source in the defined medium.

Critique of the method for characterizing lac⁺ bacteria

In view of the fact that lac⁻ bacteria in lactose medium eventually do give rise to populations which grow to completion it is necessary to specify more precisely, for operational reasons, the difference between lac⁻ and lac⁺ bacteria in their growth on lactose. By definition a lac⁺ strain consists of bacteria which, when heavily suspended in water and placed on the surface of agar containing defined medium and $6{\cdot}4 \times 10^{-4}$M (0·2 % w/v) lactose, form a heavy layer of growth in 24 hr. at 37°. Lac$^-$ bacteria, on the contrary, may form only the slightest film of background growth which, after a matter of days, becomes spotted with papillae containing lac$^+$ bacteria. When such a criterion is used to identify lac$^-$ bacteria it is found completely correlated with the formation of white to pink growth on or in Endo or EMB agar, with a long delay in the onset of growth upon inoculation into liquid medium containing lactose, and with the inability to form large concentrations of lactase within a few hours after exposure to lactose.

Despite the ability to distinguish lac$^+$ clearly from lac$^-$ bacteria upon analysis, it is still difficult to detect accurately a few lac$^+$ bacteria among many lac$^-$. The limitations of indicator agar have already been mentioned. Furthermore, when a mixed population of lac$^+$ and lac$^-$ bacteria, grown to a sugar-limited stationary phase, is plated into lactose agar, some lac$^+$ colonies, surrounded by lac$^-$ satellites, are visible to the naked eye after 1 day. With increasing time the number of lac$^+$ colonies increases. Since tenfold dilutions do not result in tenfold decreases in number of lac$^+$ colonies, we can be sure that at least some lac$^+$ colonies have resulted from the growth of lac$^+$ bacteria formed from lac$^-$ organisms in the plate. Similar results are obtained when the mixture is plated on the surface of lactose agar.

Since it is necessary to know the number of lac$^+$ bacteria present in the culture that was plated, uninfluenced by the number of lac$^+$ organisms that arise afterwards, the following experiment was performed. A series of tenfold dilutions of a sugar-starved lac$^-$ culture, with and without the addition of a fixed number of lac$^+$ bacteria, were spread on the surface of lactose agar and the number of lac$^+$ colonies was counted after 1 and 2 days. The results are shown in Table 1. It will be observed that after 1 and 2 days tenfold dilutions did not give tenfold decreases in the number of lac$^+$ colonies. When, however, the number of colonies present is plotted as a function of time and a straight line drawn through the points for a given condition and extrapolated toward zero time, tenfold decreases in the numbers of lac$^+$ colonies are found at about 18 hr. If the number of lac$^+$ colonies found at this time represents the numbers of lac$^+$ bacteria in the culture that was plated, then on the plates to which a fixed number of lac$^+$ bacteria had been added there should be an equivalent excess of lac$^+$ colonies. That this is so is shown in Table 1. Similar results were obtained on silica gels.

Rather than rely upon an extrapolation procedure the number of lac$^+$ colonies existing on the plates at 18 hr. was counted directly under a dissecting microscope. As is shown in Table 2, tenfold decreases were found and the expected number of added lac$^+$ bacteria recovered as colonies. This procedure, of demonstrated reliability, was therefore adopted as routine. The number of lac$^+$ colonies was usually counted about 16 hr. after plating. At this time there is a clear distinction between the lac$^-$ microcolonies or background growth and the lac$^+$ colonies which are then between 0·3 and 0·4 mm. in diameter. There are always, however, a few colonies of intermediate size which represent the growth of the first lac$^+$ bacteria formed on the plate, but their enumeration can be easily avoided. When twenty-five 0·1 ml. samples from the same $0{\cdot}5 \times 10^{-2}$ dilution of a lac$^-$ culture were spread on lactose agar and the lac$^+$ colonies counted after 16 hr., an average number of 15·4 with a variance of 18·7 was obtained. The probability of such a sample being taken from a homogeneous population is 0·15. During routine use of this method of counting lac$^+$ bacteria, dilutions were studied from time to time to confirm its validity.

Table 1. *The number of lac$^+$ bacteria determined by extrapolation*

Dilution of lac$^-$ culture	No. of lac$^+$ colonies			No. of excess lac$^+$ on plates to which lac$^+$ bacteria had been added
	By extrapolation (18 hr.)	By direct count (23 hr.)	By direct count (46 hr.)	(c. 227 lac$^+$ added)
10^{-3}	14	62	253	210
10^{-5}	0	0	160	240
	(16 hr.)	(22·5 hr.)	(47·5 hr.)	(c. 320 lac$^+$ added)
10^{-1}	683	733	970	379
10^{-2}	85	145	445	230
10^{-3}	6	66	355	212
10^{-4}	0	5	216	251

Table 2. *The number of lac$^+$ bacteria determined by early count*

Dilution of lac$^-$ culture	No. of lac$^+$ colonies		No. of excess lac$^+$ on plates to which lac$^+$ bacteria had been added
	(18 hr.)	(39 hr.)	(c. 22 lac$^+$ added)
10^{-2}	284	536	29
10^{-3}	21	315	20
10^{-4}	7	288	11
10^{-5}	0	99	17
	(18 hr.)	(36 hr.)	(c. 65 lac$^+$ added)
10^{-2}	579	733	—
10^{-3}	63	274	55
10^{-4}	6	160	49
10^{-5}	1	133	47

The behaviour of non-growing lac$^-$ bacteria in the presence of lactose

Although the effects of the background growth of lac$^-$ bacteria can be avoided in the enumeration of lac$^+$ organisms it remains troublesome for experiments designed to determine the fraction of the population undergoing the change. In liquid cultures it gives a turbidity that is just under the level of visibility to the naked eye. If only one mutant arose during the growth to 10^8 bacteria/10 ml. (just visible turbidity), this mutant would be expected to use whatever lactose was present and overgrow to form a lac$^+$ culture. For example, in the experiments shown in Fig. 2, at the time of the last sampling, about 2·5 % of the population in the lactose medium consisted of lac$^+$ bacteria as judged by their reaction in Endo agar. On the other hand, less than 10^{-5} of the bacteria present in the medium without lactose were lac$^+$. This could be interpreted as due to incomplete overgrowth by a small number of lac^+ mutants or due to the incomplete transformation of the lac^- bacteria, all of whom would eventually become lac^+ in the presence of lactose. One could eliminate the difficulties introduced through residual growth by further purifying the salts, by cleaning the medium of carbon source by bacterial growth with subsequent filtration, or by using bacteria whose growth is limited by the absence of some substance other than a carbon source.

When lac^- bacteria unable to synthesize tryptophan ($lac^- \, t^-$) are inoculated. into 10 ml. of medium containing lactose but devoid of tryptophan, no visible growth ensues even after 7 days (Table 3). In some instances populations as large as 10^5 bacteria/ml. developed, presumably due to contaminating traces of tryptophan, while in other instances the number of bacteria present remained essentially constant. In all, about 10^4 bacteria were tested for their lac^- character after exposure to lactose for periods varying between 1 and 7 days. The fact that, *despite a certain amount of growth in lactose*, none had been changed to a lac^+ condition suggests that the adaptation of lac^- bacteria to lactose, which would occur if the population became somewhat larger, does not involve a large fraction of the organisms present. A similar experiment allowed the same conclusion. When 5×10^3 $lac^- \, t^-$ bacteria were distributed on the surface of agar plates containing between $1{\cdot}6 \times 10^5$ and $1{\cdot}6 \times 10^3$ M lactose and layered with Endo agar after periods varying between 1 and 6 days, none became lac^+ in character.

Table 3. *The effect of lactose on* $lac^- \, t^-$ *bacteria*

Exp.	Concentration of lactose (M)	No. of bacteria/ml. found by plating in Endo agar after varying periods (hr.): 24	48	72	96	120	144	168
		(Bacteria/ml.)						
1	$1{\cdot}6 \times 10^{-3}$	196	—	214	—	4×10^3	2×10^4	—
	$1{\cdot}6 \times 10^{-5}$	214	—	236	—	376	460	—
	0	212	—	242	—	262	290	—
2	$1{\cdot}6 \times 10^{-3}$	136	144	154	230	413	356	270
	$1{\cdot}6 \times 10^{-4}$	154	154	118	118	144	123	101
	$1{\cdot}6 \times 10^{-5}$	125	126	156	233	341	> 500	> 500
	$1{\cdot}6 \times 10^{-6}$	115	156	122	111	186	55	53
	0	131	274	770	3×10^3	—	—	5×10^3

Apparently under these conditions the adaptation of lac^- bacteria in the presence of lactose involves very few members of the population. It could conceivably be argued, however, that in the presence of limiting concentrations of tryptophan (or of any other substance) the enzyme syntheses required to make all or most of the cells lac^+ cannot occur. To take this possibility into account it is necessary to examine by some other method the question of whether the change to lac^+ occurs only in special members of the population. This question will be further considered under Discussion.

The effect of lactose on the rate of mutation from the lac$^-$ to the lac$^+$ condition

The bacteria in a lac$^-$ population that are capable of utilizing lactose for growth may have an origin that is independent of, or dependent upon, that sugar. If the latter were true the chance that a lac$^-$ bacterium would be converted into a lac$^+$ would be different in the presence and absence of lactose. There are several ways to determine the magnitude of this chance.

Advantage may be taken of the fact that lac$^-$ microcolonies form lac$^+$ papillae. Since the papillae are usually well separated on the colony surface each one must represent at least one independently mutated lac$^-$ bacterium. Because of the delay between mutation and the formation of a visible papilla, and because, due to their location, some mutant bacteria may be unable to manifest their presence, the papilla count will always be lower than the number of mutations. Hence the mutation rate calculated by this method is a minimum estimate. In one experiment, in the presence of 6×10^{-4} M lactose an average of 3·9 papillae was found among 70 microcolonies whose average bacterial content was $1{\cdot}7 \times 10^7$. The total number of bacterial generations involved in the formation of a microcolony of this size is $\frac{1{\cdot}7 \times 10^7}{\ln 2}$ and this divided into 3·9 gives $1{\cdot}6 \times 10^{-7}$ as the chance per generation that any lac$^-$ bacterium will mutate to a lac$^+$.

The same rate of change is found when, by the Poisson formulation, the average number of papillae is calculated from the number of colonies without papillae. It is first necessary to show that the papillae are distributed at random among lac$^-$ colonies. For this purpose Endo agar and agar containing $1{\cdot}3 \times 10^{-3}$ MD-galactose and $6{\cdot}4 \times 10^{-4}$ M lactose were chosen since they supported considerable growth of the lac$^-$ bacteria, thus allowing a reasonable number of papillae to form. In measuring this distribution special care was taken to use agar plates of uniform thickness and to consider only well-formed separated colonies of the same size. The results are shown in Table 4. The evidence is consistent with the hypothesis that papillae (and hence the mutations they represent) are distributed at random among the colonies.

Table 4. *The distribution of mutant papillae on colony surfaces*

Medium	Colony diameter (mm.)	No. of colonies	Average no. of papillae/colony	Probability of homogeneity	Probability of drawing a similar sample from Poisson distribution
Endo agar	3·4	35	10·0	0·3	0·5
Lactose-galactose agar	2·2	55	2·0	$\gg 0{\cdot}05$	0·4
	2·4	34	2·9	0·3	0·7
	2·5	20	3·0	0·5	0·07

Table 5 summarizes the mutation rates obtained by the calculation by the Poisson method of the number of papillae among about 10^4 microcolonies and shows that the rate of change is independent of the concentration of lactose used and of the size of the colonies in which the change occurs. The data on which this table is based are homogeneous by χ^2 test ($P = \geqslant 0{\cdot}05$) and give an average chance of change of $1{\cdot}6 \times 10^{-7}$/bacterium/generation in the presence of lactose.

Table 5. *The rate of mutation to lac$^+$/generation of lac$^-$ bacteria as a function of the concentration of lactose and the size of the microcolony*

Concentration of lactose (M)	Rate of change	No. of bacteria in microcolony	Rate of change
$1{\cdot}6 \times 10^{-3}$	$2{\cdot}0 \times 10^{-7}$	$6{\cdot}4$–$9{\cdot}9 \times 10^{4}$	$1{\cdot}3 \times 10^{-7}$
$8{\cdot}0 \times 10^{-4}$	$1{\cdot}1 \times 10^{-7}$	$1{\cdot}0$–$4{\cdot}9 \times 10^{5}$	$2{\cdot}3 \times 10^{-7}$
$1{\cdot}6 \times 10^{-4}$	$1{\cdot}4 \times 10^{-7}$	$5{\cdot}0$–$9{\cdot}9 \times 10^{5}$	$2{\cdot}0 \times 10^{-7}$
$1{\cdot}3 \times 10^{-4}$	$0{\cdot}94 \times 10^{-7}$	$1{\cdot}0$–$4{\cdot}9 \times 10^{6}$	$1{\cdot}3 \times 10^{-7}$
$3{\cdot}0 \times 10^{-5}$	$1{\cdot}1 \times 10^{-7}$	$5{\cdot}0$–$8{\cdot}6 \times 10^{6}$	$1{\cdot}1 \times 10^{-7}$
$1{\cdot}6 \times 10^{-5}$	$1{\cdot}8 \times 10^{-7}$	—	—

It may seem surprising that the minimum estimate of the rate of mutation does not increase as the rate of growth of the colony slows down for this should allow a relatively faster rate of papilla formation in comparison to the rate of formation of new lac$^-$ organisms and mutants among them. In fact, the rate of papilla development (growth of lac$^+$ bacteria) is undoubtedly so fast in the region of exponential growth of the lac$^-$ microcolonies (Fig. 1) as to bring the calculation of minimum mutation rate very close to the true rate. It has not been possible to measure the actual rate of growth of lac$^+$ bacteria in papillae for technical reasons, but if it were 10 times as fast as the growth of the microcolony (0·06 doubling/hr.), an assumption that semi-quantitative observation indicates is quite possible, the minimum mutation rate calculated would be indistinguishable from the true rate. This approximation is also not affected by death of bacteria in the central non-growing portion of the colony, for total and viable counts of the number of bacteria in microcolonies are indistinguishable.

Another method of calculating the mutation rate in the presence of lactose involves the use of the data described in Fig. 2 (see p. 74). At the time of the last sampling of the cultures containing lactose some lac$^+$ bacteria were found to be present. If these bacteria are assumed to have been in the process of overgrowing their parental lac$^-$ population, which was in a stationary phase because of exhaustion of the carbon source, then it is possible to estimate the time of origin of the first lac$^+$ bacterium by assuming that it multiplied at the maximum rate the medium would allow (1·2 generations/hr.). (The rate of increase of at least some mutants in growing cultures of their parents seems to be almost exponential; Ryan & Schneider, unpublished.) The number of lac$^-$ bacteria present at the time of origin of the first lac$^+$ can be read directly from the growth curve and when divided by ln 2 we obtain the number of bacterial generations after which the first mutation occurred. The chance of the mutation is the reciprocal of this, and in the case of the two experiments shown in Fig. 2 was $2{\cdot}4 \times 10^{-7}$ and $2{\cdot}8 \times 10^{-7}$/bacterium/generation. These figures are good approximations of the result obtained by the papilla method.

The chance of appearance of a lac^+ bacterium in the absence of lactose can be determined by using one of a number of methods, the most convenient of which for our purposes is the median method of Lea & Coulson (1949). This involves finding the median number of new types in a series of similar cultures begun with a very small number of the parental type. For this purpose, in two experiments about 1000 lac^- bacteria were inoculated into 10 ml. medium containing a limiting amount of maltose. Fifty-four 0·1 ml. samples were then distributed to small test-tubes which were incubated in a moist chamber. After growth was complete the contents were removed with a pipette, and tubes were rinsed twice with 0·1 ml. lots of water. The contents of four of the tubes were diluted and plated in maltose agar to determine the total bacterial number. The whole contents of the remaining fifty tubes were spread on the surface of lactose agar to determine the number of lac^+ bacteria. The results in Table 6 show that in the absence of lactose the chance of appearance of lac^+ bacteria per lac^- bacterium per generation is about $2{\cdot}6 \times 10^{-7}$. This rate is indistinguishable from that observed in the presence of lactose.

Table 6. *Variance analysis of the numbers of lac⁺ mutants in different cultures in the absence of lactose*

Concentration of maltose (M)	Total no. of bacteria/ 0·1 ml.	Median no. of lac^+ bacteria	Chance of mutation/lac^- bacterium/ generation	Mean no. of lac^+ bacteria	Variance of the mean	Probability that the sample was from a homogeneous population of cultures
$5{\cdot}6 \times 10^{-4}$	$3{\cdot}1 \times 10^{7}$	38·5	$2{\cdot}1 \times 10^{-7}$	55	2597	$<0{\cdot}01$
$5{\cdot}6 \times 10^{-4}$	$3{\cdot}4 \times 10^{7}$	45	$2{\cdot}7 \times 10^{-7}$	54	5510	$<0{\cdot}01$
$1{\cdot}8 \times 10^{-4}$	$1{\cdot}3 \times 10^{7}$	12·5	$2{\cdot}5 \times 10^{-7}$	24	2386	$<0{\cdot}01$
$1{\cdot}4 \times 10^{-3}$	$0{\cdot}9 \times 10^{-6}$	0	$3{\cdot}2 \times 10^{-7}$	0·9	2·6	$<0{\cdot}01$

Variance analysis of the numbers of lac⁺ bacteria appearing in different cultures in the absence of lactose

It might be argued that the reason a lac^+ bacterium has the same chance of appearing in the presence or in the absence of lactose is the fact that the mutants presumed to have been formed in maltose medium were counted after growing on plates in the presence of lactose. To test this a variance analysis of the data was made as suggested by Luria & Delbrück (1943). Table 6 shows that the probability that the cultures utilized came from a homogeneous population of cultures is extremely small.

In contrast with the observed results the notion that exposure to the lactose in the testing plates induces the formation of lac^+ bacteria demands that cultures of lac^- bacteria growing in maltose be homogeneous with regard to their ability to respond to lactose. Jackson & Hinshelwood (1949) offered a reason why cultures homogeneous in this regard would appear to be heterogeneous when plated on a testing agar. Their argument assumes that upon plating lac^- bacteria on lactose rapid death occurs among those that have not responded to lactose by becoming lac^+. The rate at which death occurs is assumed to be a function of the length of time in which the culture has been in the stationary phase before plating, and it would be expected that this would vary somewhat from culture to culture. Such a possibility does not exist in these experiments, for not only does the number of lac^- and lac^+ bacteria remain constant for more than 24 hr. in lac^- cultures whose growth has ceased in limiting amounts of maltose, but each lac^- bacterium plated on lactose agar, rather than dying, survives and forms a microcolony.

Nor is it probable that the observed variation was introduced by fluctuation in the manipulations employed in the variance analysis experiment (Eriksen, 1949). In the course of incubation the cultures lost an average of only 8 % ($\sigma = 6$ %) of their weight. In order to test whether the procedure employed removed all the bacteria in the small cultures the following experiment was performed. Twenty-five 0·1 ml. lots of medium, each containing about 25 bacteria, were pipetted into tubes from which they were immediately washed out by the usual procedure. Twenty-five other samples were plated directly from the source as a control. Both series were homogeneous by χ^2 test ($P = 0{\cdot}7$ and $0{\cdot}2$ respectively) thereby confirming the accuracy of pipetting 0·1 ml. lots of medium. The means were 25 and 27, showing a difference that is not significant ($t = 1{\cdot}4$, $P = 0{\cdot}2$) and proving that essentially all of the culture was washed out. Furthermore, the spreading procedure used for the detection of lac^+ bacteria did not result in an appreciable loss of bacteria as was shown by plating 10 cultures containing about 70 bacteria on the surface of agar and 10 cultures directly in agar. In the former case the average number of colonies was 69, while an average of 72 was obtained in the latter case. Once again the difference was not significant ($t = 0{\cdot}87$, $P = 0{\cdot}4$). The counts of the total number of bacteria in the 0·1 ml. cultures showed a small variation, σ never exceeding 25 %. As already mentioned, the method of enumerating lac^+ bacteria showed upon test a variance not unexpected in terms of normal sampling error.

The most likely reason for the heterogeneity in numbers of lac^+ bacteria among different cultures is that, if mutations have a fixed chance of occurring during growth in the absence of lactose, some will occur earlier than others and be the origin of greater numbers of mutants. If this be true the distribution of mutant numbers should not only have a large variance but it should have the specific form of the distribution calculated by Lea & Coulson (1949). The data from the experiments reported in Table 6 fit very well the theory of Lea & Coulson. Fig. 3 shows a comparison of the observed and theoretical distributions of the number of lac^+ mutants in different cultures containing $5{\cdot}6 \times 10^{-4}\,\text{M}$ lactose. The probability that the two distributions come from a homogeneous population is 0·2. Similar results were obtained with smaller populations grown on $1{\cdot}8 \times 10^{-4}$ ($P = 0{\cdot}3$) and $1{\cdot}4 \times 10^{-3}$ ($P = 0{\cdot}3$) M maltose. Since this distribution has a unique form it is quite unlikely that the fit is coincidental. Rather, the good correspondence can be taken to provide a final demonstration that the lac^+ bacteria found on the lactose agar plates arose at random during the growth of their lac^- parents in maltose and did not arise on the lactose agar plates. Hence, the agreement between mutation rates in the presence and absence of lactose establishes the fact that the lac^- to lac^+ mutation is independent of this sugar.

The extent to which the lac⁻ gene is independent of its chemical environment in mutating to the lac⁺ condition

Since it is conceivable that, despite independence of lactose, mutation of the lac$^-$ gene may be dependent upon one or another related compounds, several substances were tested by the methods already described. The first involved the use of lac$^-$t$^-$ bacteria in liquid medium and on the surface of agar. It was at the same time possible to test mutation in the reverse direction by the use of lac$^+$t$^-$. Table 7 summarizes the results obtained. Sporadic and limited growth of the sort shown in Table 3 occurred, but there was no evidence of an augmentation of mutant frequency. Among the lac$^-$t$^-$ bacteria no lac$^+$ mutants were evident, while the single lac$^-$ mutant found among the lac$^+$t$^-$ parents in the presence of galactose may well be of spontaneous origin.

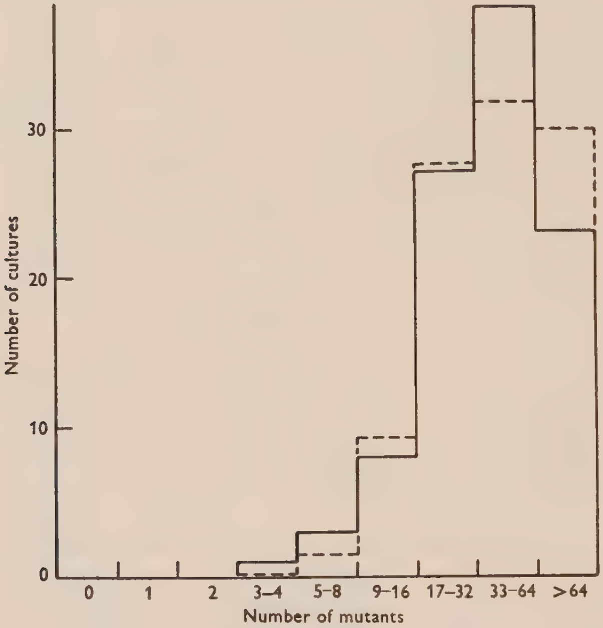

Fig. 3. The distribution of lac$^+$ mutants in strain ML 3*a* of *Esch. coli*. The solid line represents the observed distribution; the dotted line the theoretical distribution calculated according to Lea & Coulson (1949) on the basis of an average number of mutations determined from the median of the observed distribution.

The same substances were also tested for their effect upon the rate of mutation in microcolonies but, as is shown in Table 8, there is no significant difference between the rate of mutation in their presence and in their absence. Both of the above methods were chosen as affording the least opportunity for irrelevant selection phenomena to obscure the results. Hence, in terms of mutability the lac$^-$ locus appears to be extremely insulated from its chemical environment.

Table 7. *The numbers of bacteria tested for a modified genotype in the presence of a variety of compounds in liquid medium and on the surface of agar*

		Strain lac$^-$t$^-$		Strain lac$^+$t$^-$	
Substance added	Concentration (M)	No. of bacteria examined	Mutant frequency unlikely to be more than*	No. of bacteria examined	Mutant frequency unlikely to be more than*
Lactose	$1{\cdot}6 \times 10^{-3}$	4572	2×10^{-4}	1377	7×10^{-4}
	$1{\cdot}6 \times 10^{-6}$	10636	1×10^{-4}	2450	4×10^{-4}
D-Galactose	$3{\cdot}2 \times 10^{-3}$	2902	3×10^{-4}	2784†	4×10^{-4}
	$3{\cdot}2 \times 10^{-6}$	8581	1×10^{-4}	4285	2×10^{-4}
Phenyl-β-D-thio-galactoside	$1{\cdot}0 \times 10^{-3}$	1566	6×10^{-4}	1648	6×10^{-4}
	$1{\cdot}0 \times 10^{-6}$	6494	2×10^{-4}	6763	1×10^{-4}
Methyl-β-D-galactoside	$1{\cdot}5 \times 10^{-4}$	2224	5×10^{-4}	2344	4×10^{-4}
Melibiose	$1{\cdot}5 \times 10^{-4}$	2609	4×10^{-4}	2165	5×10^{-4}
D-Fucose	$3{\cdot}0 \times 10^{-3}$	2019	5×10^{-4}	2538	4×10^{-4}
L-Sorbose	$2{\cdot}8 \times 10^{-3}$	1324	8×10^{-4}	1204	8×10^{-4}
Nil	—	8120	1×10^{-4}	6098	2×10^{-4}

* Approximate reciprocal of previous column.

† One lac$^-$ mutant found; in no other experiment were any mutants found.

Table 8. *Mutation rates to lac$^+$ in microcolonies grown on agar in the presence of lactose and other compounds*

Concentration of lactose (M)	Substance added	Concentration (M)	Chance of mutation/bacterium/generation
3×10^{-5}	D-Galactose	$3{\cdot}2 \times 10^{-3}$	$0{\cdot}64 \times 10^{-7}$*
3×10^{-5}	D-Galactose	$3{\cdot}2 \times 10^{-4}$	$2{\cdot}60 \times 10^{-7}$
3×10^{-5}	D-Galactose	$3{\cdot}2 \times 10^{-5}$	$0{\cdot}97 \times 10^{-7}$
3×10^{-5}	D-Galactose	$3{\cdot}2 \times 10^{-6}$	$0{\cdot}73 \times 10^{-7}$
$1{\cdot}3 \times 10^{-4}$	Phenyl-β-D-thio-galactoside	1×10^{-3}	$1{\cdot}4 \times 10^{-7}$
$1{\cdot}3 \times 10^{-4}$	Methyl-β-D-galactoside	$1{\cdot}5 \times 10^{-4}$	$1{\cdot}9 \times 10^{-7}$
$1{\cdot}3 \times 10^{-4}$	Melibiose	1×10^{-3}	$1{\cdot}3 \times 10^{-7}$
$1{\cdot}3 \times 10^{-4}$	D-Fucose	$1{\cdot}5 \times 10^{-3}$	$1{\cdot}1 \times 10^{-7}$
$1{\cdot}3 \times 10^{-4}$	L-Sorbose	$1{\cdot}4 \times 10^{-3}$	$0{\cdot}77 \times 10^{-7}$

* $0{\cdot}49 \times 10^{-7}$ by Poisson method, $0{\cdot}78 \times 10^{-7}$ by count of the number of papillae.

DISCUSSION

The case of the adaptation of lac$^-$ bacteria to use lactose is instructive, inasmuch as the complete expression of the mutation involves both the development of an enzyme in response to a substrate and the selective overgrowth of a mutant able to form this enzyme. The mutation to the lac$^+$ condition occurs independently of lactose in a random fashion and simply confers the capacity to respond to lactose by the adaptive formation of lactase. In the absence of lactose competent lac$^+$ organisms cannot be distinguished from lac$^-$; both possess the same basal amount of β-galactosidase. When lactose is present, on the other hand, the rare lac$^+$ bacteria can complete the adaptive response by developing lactase and using the substrate. As a result the lac$^+$ bacteria have a selective advantage and overgrow their parental lac$^-$ bacteria, bringing about a heritable change in the population. A mutation rate of the magnitude found in the absence of lactose allows the calculation of exactly the amount of overgrowth of lac$^+$ bacteria found in the experiments referred to in Fig. 2. Where mutations result in the capacity to form enzymes that are not adaptive, adaptations take place by selection without the substrate (or its absence) provoking either the adapted genotype or the phenotype it allows. In this case the environment acts solely as a selective agent (Ryan & Schneider, 1949; Fildes & Whitaker, 1948).

Hinshelwood (1950), in his theory of bacterial adaptation, has failed to assess the role which the origin of genetic competence plays in this phenomenon. By the use of physical chemical models he has elaborated systems in which enzyme concentrations vary in accordance with the chemical environment and has proposed that such systems account for bacterial adaptation. Following Hinshelwood's line of interpretation, the enzyme, lactase, would be expected to increase autocatalytically in individual lac$^-$ bacteria due to the presence of the substrate, lactose, and the bacterial population would by this means be transformed into the lac$^+$ condition. The error here lies in the failure to recognize the nature of the genetic difference between lac$^+$ and lac$^-$ bacteria and the random character of its origin.

There is no question about the existence of adaptively formed enzymes, but we may ask whether there is evidence that *inherited* adaptations are ever brought about by the mechanism proposed by Hinshelwood. The publications of Hinshelwood and his school on this problem are so numerous as to preclude any detailed assessment in this discussion. However, it is possible to evaluate the arguments against the mutational origin of adaptation that they have used in their work. These have conveniently been summarized by Hinshelwood (1950).

The first objection refers to the conclusions that have been drawn by others from variance analyses of the numbers of mutants in different cultures. Hinshelwood has suggested that the results may not be in agreement with the hypothesis of induced change for reasons other than that they were brought about by random mutations. This is a legitimate objection but cannot apply to the present case for the reason that there is an exact *quantitative* agreement between the actual distribution of mutants and that predicted by random mutation. This distribution is not expected by any alternative hypothesis thus far proposed. For the same reason the objection is not valid in the case of a number of other adaptations. In several instances, however, the observed and theoretical distributions are not in accord, but even in these cases the reason for the disagreement may be consistent with the mutation hypothesis (Ryan, 1952). Furthermore, the specific, non-mutational interpretations that have been offered by Jackson & Hinshelwood (1949) and by Eriksen (1949) to account for the extremely large variance found in mutant distributions have been shown in the present case to be inapplicable. As a consequence, it can be concluded that where variance analyses have been properly carried out the evidence has been in accord with the hypothesis of mutation and is inconsistent with the notion of adaptively induced change.

The remaining objections to the mutation hypothesis arise especially from the findings of Hinshelwood's school (see Dean & Hinshelwood, 1951). In the first place they frequently found that very small inocula adapt and consequently concluded that all of the bacteria introduced into the new environment must be involved in the change. A similar conclusion was drawn by Virtanen (1948) who observed that every organism of *Esch. coli* plated on the surface of agar adapted to use lactose. In both of these cases it is necessary to determine whether the responsible factor might have been the slow, background growth of parental types which resulted in the formation of mutants that were selected for. Jackson & Hinshelwood (1949), for example, studying the adaptation of *Esch. coli* to use lactose, reported that agar and silica gel, to which no carbon source had been added, supported the slow growth of colonies. This fact, which we have confirmed, serves to invalidate their conclusion that the adaptation did not involve mutation. Where background growth cannot be avoided its effect must be estimated before a statement can be made as to whether or not each of the original bacteria becomes adapted.

Another argument against the mutation hypothesis involves the fact that, if mutation is the basis of the adaptation being studied, forward and back mutation rates (in addition to whatever specific selection operates for or against the mutant) should eventually bring about an equilibrium in a population growing in the absence of the substance with which the adaptation is concerned. Hinshelwood & Jackson (1950) found that the serial transfer of *Aerobacter aerogenes* in various media did not decrease the lag period required for growth on D-arabinose. It was concluded that the populations must have been at equilibrium with regard to the proportion of arabinose positive mutants (which exhibit a very short lag period on arabinose). The equilibrium frequency of arabinose-adapted organisms was calculated to be about 10^{-7}, indicating that the rate of mutation from ability to inability to use arabinose (or selection against arabinose positive organisms) must be rather high. Nevertheless, when arabinose positive cultures were serially transferred in the absence of arabinose they remained stable indefinitely. This evidence would seem to indicate that the mutation hypothesis cannot account for the behaviour of this bacterial system.

A similar result was observed in populations of *Esch. coli* mutating from L-histidine dependence to independence (Atwood, Schneider & Ryan, 1951). The explanation was found, however, to be completely consistent with the mutation hypothesis. In the course of serial transfer, mutations to faster growing types appeared which had no relation to the mutation to histidine independence that was being studied. These fitter types overgrew the old population and eliminated not only the parental bacteria but also the mutants among them. Eventually among the fitter bacteria new mutants appeared but these too were eliminated when a further fitter type appeared in the culture. In this way, since periodic selection occurred frequently enough, the mutants were prevented from attaining true mutational equilibrium and a pseudoequilibrium resulted in which mutant frequency was much lower. Since the mutation to faster growth almost invariably occurs in the major component of the population, periodic selection serves to stabilize that component during growth. Yet there is always present a small reserve of mutant organisms which can overgrow the population when the environment changes in their favour. This phenomenon appears to be general and, unless it is shown not to operate in a particular system, the failure of mutational equilibrium to be obtained cannot be used as evidence that mutation is not the basis of the change being observed.

Hinshelwood has placed much weight on the claim that the stability of an adaptation is a function of the 'thoroughness' of training. Incompletely trained organisms lose their adaptation upon removal from the medium to which they are adapted. But completely trained organisms, after long exposure to the medium in question, are stable when removed from it. This could be interpreted as due to the fact that incompletely trained cultures, although consisting mostly of mutants, still contained a few unadapted parental bacteria which overgrow when transferred to another medium. Cooke & Hinshelwood (1947) (see also Pryce & Hinshelwood, 1947) tested this possibility by reconstructing a mixture of *A. aerogenes* consisting of bacteria unable to utilize glycerol rapidly and stable organisms that had been completely 'trained' to use this substance by a large number of transfers in its presence. When this mixture was serially transferred on sugar it did not change to a glycerol non-utilizing condition, a fact that convinced the investigators that incompletely trained cultures could not consist of such mixtures. This conclusion does not necessarily follow since the adapted bacteria, obtained after many generations of training, might well have been faster growing types that came to predominate by a periodic selection of the sort described above. Although non-adapted bacteria may have a selective advantage over adapted bacteria of their own growth type they would not possess such an advantage over adapted bacteria of a fitter type. Reconstruction experiments of the sort performed by Cooke & Hinshelwood can be instructive only when they involve components of the same growth type.

Finally Hinshelwood, calling attention to the fact that adaptations may be additive, contends that on the mutation hypothesis 'the role assigned to chance is a rather remarkable one'. Be that as it may, we should not fail to appreciate the powerful directive role of the environment, not in inducing mutations, but in determining their effect on the constitution of the population. This, together with a recognition of the pleomorphic effect of certain genes, might even lead one to anticipate more plasticity on the part of populations than has been hithertofore demonstrated.

The above considerations, taken in conjunction with those presented by Cavalli (1951) on bacterial adaptation to drug resistance, lead to the conclusion that no solid evidence for Hinshelwood's theory exists. This, of course, does not exclude the possibility that in some instances, when the proper experimental tests have been performed, his theory will provide the best interpretation. But, before it is accepted in any instance, modern genetic conceptions must be shown clearly not to apply.

We are left then with the result that some adaptations, at least, originate in the form of random mutations. It is not so surprising that mutations, under constant environmental conditions, are distributed in time and in space at random. But one may wonder why, if a gene is a differentiated molecule (or part of a molecule), it has not been possible to cause it to react in a specific way and thereby mutate. Chemical mutagens are well known but they do not cause patterns of mutation different from those induced by irradiation (Tatum & Perkins, 1950).

Compounds related to the activity of the lac$^+$ locus were chosen as possible mutagens for this study, on the working hypothesis that genes may not be unrelated in configuration to the enzymes whose synthesis they control, and hence may enter into similar chemical combinations (see, for example, Emerson, 1945).

The compounds used have been shown by Monod *et al.* (1951) to have certain relations to the activity of the lac$^+$ locus. Lactose, for example, can be used as a carbon source by lac$^+$ bacteria, is a good inducer of β-galactosidase in these organisms, and has a strong affinity for the enzyme *in vitro*. In the same strain, D-galactose is a moderate inducer of lactase and has a feeble affinity. Phenyl-β-D-thiogalactoside, on the other hand, although it is inactive as an inducer, has a strong affinity for the enzyme, acts as a competitive inhibitor of its activity and prevents the formation of the enzyme. In contrast, methyl-β-D-galactoside and melibiose are excellent inducers but have little or no enzyme affinity. Fucose (6-deoxygalactose), like the unrelated sugar, sorbose, neither induces nor has affinity. If a gene concerned with the synthesis of lactase possessed groups similar to those causing the affinity of lactase for some of the compounds tested, the combination of the gene with these substances would be expected to have modified its stability. Monod *et al.* (1951) showed that the act of induction does not involve the combination of the inducer, or a derivative of it, with the enzyme formed. If the act of induction involved a combination with the gene for reasons other than an affinity for lactase, some of these substances might have entered into such combinations. The fact that none of these compounds significantly altered genetic stability does not, however, provide a rigorous test of the hypothesis of the similarity between gene and enzyme. In the first place, the mutated lac$^-$ gene may not be able to enter into the same combinations as the lac$^+$ gene so that only the experiments involving the detection of lac$^-$ mutants among lac$^+$ parents may be relevant. This may be especially true if the lac$^-$ strain is what it is because it possesses a suppressor mutation at some locus only indirectly concerned with lactase synthesis. Further, the similarity between gene and enzyme may not involve the groups with which the substances tested enter into combination. The negative results obtained should not preclude further examination of the notion. In any event the results would seem to indicate that the gene is not promiscuous in its combination with chemicals in its environment.

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REFERENCES

Atwood, K. C., Schneider, L. K. & Ryan, F. J. (1951). Periodic selection in *Escherichia coli*. *Proc. nat. Acad. Sci., Wash.* **37**, 146.

Barer, G. R. (1950). The action of streptomycin on *Bacterium lactis aerogenes*. *J. gen. Microbiol.* **4**, 1.

Cavalli, L. (1951). Genetical analysis of drug resistance. *Symp. Int. Chim. Microbiol. Roma*, no. 101.

Cohn, M. & Monod, J. (1951). Purification et propriétés de la β-galactosidase (lactase) d'*Escherichia coli*. *Biochim. Biophys. Acta*, **7**, 153.

Cooke, E. G. & Hinshelwood, C. N. (1947). The adaptation of *Bacterium lactis aerogenes* to glycerol and to various carbohydrates. *Trans. Faraday Soc.* **43**, 733.

Dean, A. C. R. & Hinshelwood, C. N. (1951). Induced and other variations in bacterial cultures. *J. chem. Soc.* p. 1157.

Emerson, S. (1945). Genetics as a tool for studying gene structure. *Ann. Mo. bot. Gdn.* **32**, 243.

Eriksen, K. R. (1949). Studies on the mode of origin of penicillin resistant Staphylococci. *Acta path. microbiol. scand.* **26**, 269.

Fildes, P. & Whitaker, K. (1948). 'Training' or mutation of bacteria. *Brit. J. exp. Path.* **29**, 240.

Hinshelwood, C. N. (1950). Chemistry and bacteria. *Nature, Lond.* **166**, 1089.

Hinshelwood, C. N. & Jackson, S. (1950). The stability of D-arabinose adaptation of *Bact. lactis aerogenes*. *Proc. Roy. Soc.* B, **137**, 88.

Jackson, S. & Hinshelwood, C. N. (1949). An investigation of the nature of certain adaptive changes in bacteria. *Proc. Roy. Soc.* B, **136**, 562.

Lea, D. E. & Coulson, C. A. (1949). The distribution of the numbers of mutants in bacterial populations. *J. Genet.* **49**, 264.

Luria, S. E. & Delbrück, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics*, **28**, 491.

Massini, R. (1907). Über einen in biologischer Beziehung interessanten Kolistamm (*Bacterium coli mutabile*). *Arch. Hyg., Berl.* **61**, 250.

Monod, J. & Audureau, A. (1946). Mutation et adaptation enzymatique chez *Escherichia coli-mutabile*. *Ann. Inst. Pasteur*, **72**, 868.

Monod, J., Cohen-Bazire, G. & Cohn, M. (1951). Sur la biosynthèse de la β-galactosidase (lactase) chez *Escherichia coli*. La specificité de l'induction. *Biochim. Biophys. Acta*, **7**, 585.

Monod, J. & Cohn, M. (1952). La biosynthèse induite des enzymes (Adaptation enzymatique). *Advances in Enzymology* (in the Press).

Pryce, J. M. & Hinshelwood, C. N. (1947). The adaptation of *Bacterium lactis aerogenes* to various inhibitors. *Trans. Faraday Soc.* **43**, 742.

Ryan, F. J. (1952). The distribution of numbers of mutant bacteria in replicate cultures. *Nature, Lond.* **169**, 882.

Ryan, F. J. & Schneider, L. K. (1949). The consequences of mutation during the growth of biochemical mutants of *Escherichia coli*. IV. The mechanism of inhibition of histidine independent bacteria by histidineless bacteria. *J. Bact.* **58**, 201.

Tatum, E. L. & Perkins, D. D. (1950). Genetics of microorganisms. *Ann. Rev. Microbiol.* **4**, 129.

Virtanen, A. I. (1948). Adaptive formation of enzymes. *Svensk kem. Tidskr.* **60**. 23.

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Williams, R. E. O. (1952). *J. gen. Microbiol.* 7, 89–97

Investigations into a Method for Counting the Total Number of Bacteria in a Suspension

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SUMMARY: Counts were made of the total number of bacteria in cultures by a method that comprised suspension of the cells in molten agar, and the casting of this agar into a thin film in a haemocytometer chamber, followed by removal of the film from the chamber and drying in air. Counting of the bacteria was carried out with the use of a phase-contrast microscope. Statistical analysis of the counts indicated that the principal sources of variation lay in the differences between the numbers of organisms counted in successive microscope fields, between the total numbers in different traverses of the film, and between the primary agar dilutions of the culture.

In many experiments where a count of the total number of bacteria in a suspension is required, it would be an advantage if the actual counting could be postponed until the rest of the experiment had been concluded. This is the case, for example, when the suspension has to be used immediately for the absorption of serum or inoculation into animals. The most precise method of making total counts is generally considered to be by the use of a counting chamber such as that described by Helber (1904; see *Topley and Wilson's Principles*, 1946), but the method described in another connexion by Jones & Mollison (1948) in which permanent preparations in agar films are made for counting might have some advantages when postponement of the counting is required, and it seemed worth further investigation. A number of tests of the precision of this method were therefore made.

METHOD

The procedure finally adopted for making the preparations and counting the bacteria was as follows:

(1) One millilitre quantities of 1·9 % agar in normal saline are distributed in plugged test-tubes 11 mm. diam. and held molten in a water-bath at 60–65°. (One batch of New Zealand agar was used throughout the present study.)

(2) The bacterial suspension is mixed well and, with a calibrated Pasteur pipette, 0·2 ml. is transferred to 1 ml. agar.

(3) By means of a fresh pipette, the agar suspension is mixed well and a drop run into one side of a double Neubauer haemocytometer chamber (0·1 mm. deep) fitted with the standard cover slip; the suspension is mixed again, and a drop run into the other side of the chamber. The glass chamber should be slightly warmed before use so that the agar flows readily.

(4) The agar is allowed to set, which takes about 5 min., and the chamber is then immersed in cold water in a dish. The cover-slip is raised gently by inserting a straight wire into the side trough and the two agar films then

usually float easily off the glass slide. The films are caught on glass microscope slides and removed from the water. The transfer is facilitated by the use of a dish with a black bottom, strongly illuminated from one side.

(5) The agar films on the slides are trimmed and then allowed to dry in air, and are then mounted under a cover-slip in glycerol jelly.

(6) Counting of the bacteria in the films is carried out with a phase-contrast microscope, using a $\frac{1}{12}$ in. oil-immersion objective and a ×6 eyepiece, with a square mask. Some difficulty may be experienced in counting from the fact that not all the organisms lie in one focal plane; it may be easier therefore to use a $\frac{1}{6}$ in. or a $\frac{2}{3}$ in. objective and a higher power compensating eyepiece.

(7) The number of bacteria per cu.mm. of the agar suspension is calculated from the number per microscope field by using a factor derived from the area of the field in sq.mm. (as determined by a stage micrometer) multiplied by 0·1, the depth of the agar in the haemocytometer chamber.

TESTS OF THE METHOD

During the preliminary investigations of the method suspensions of streptococci were used, as it was for these organisms that a counting method was to be used, but since the results of many partial tests of the various stages in the technique were amply confirmed in two final comprehensive tests these alone will be described in detail. For one of these a suspension of *Streptococcus pyogenes*, Type 3 (strain Richards), was used, and for the second a suspension of *Bacterium coli* (strain D 433). The latter was a culture grown at 37° for about 12 hr. and was almost entirely in the form of single cells; the former had a median chain length of 6·3 cocci. Since the existence of such chains naturally complicates the analysis, the results from the experiment with the *Bact. coli* suspension will be given first.

Eight agar dilution tubes were melted and cooled to 60°. With a '50-drop' pipette a sample was taken from the suspension and 0·2 ml. delivered into each of two tubes of agar. With a fresh Pasteur pipette the agar dilution was well mixed and drops were run into the two sides of the counting chamber. Using another pipette this subsampling was repeated. The films were floated off and mounted.

Four traverses, each consisting of the examination of five fields, were made on each film. In each traverse the five fields were spaced more or less equally along either a long- or a cross-axis of the film. The whole process was repeated four times so as to give a total of 32 films. The examination of the 32 films was carried out in a random order.

The structure of the experiment was thus as follows:

Samples with 50-drop pipette from culture $(S)=4$.

Dilution tubes for each sample $(D)=2$, i.e. total number of dilution tubes $(D')=8$.

Subsamples from each dilution tube $(U)=2$, i.e. total subsamples $(U')=16$.

Films from each subsample $(F)=2$, i.e. total films $(F')=32$.

Traverses of each film $(T)=4$, i.e. total traverses $(T')=128$.

Fields examined in each count $(L)=5$, i.e. total fields $(L')=640$.

The frequency distribution of the counts of bacteria per field was found to be indistinguishable from a normal distribution (Fig. 1, curve *A*). On general principles one would expect a Poisson form of frequency distribution in this case, but with a mean number of cells per field of 26·69 the two distributions are very similar in shape, though the true Poisson distribution has a smaller standard deviation (Fig. 1, curve *B*).

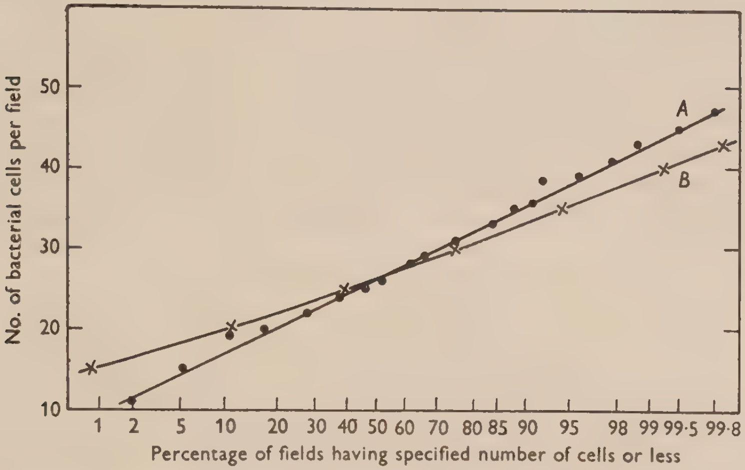

Fig. 1. Frequency distribution of numbers of *Bact. coli* per microscope field (*A*), compared with that to be expected from a population having a Poisson distribution with approximately the same mean value (*B*). Curves drawn on a probability scale for the abscissa, such that a normal distribution is represented by a straight line.

Table 1. *Analysis of variance of counts of* Bact. coli

Source of variance	Sums of squares	Degrees of freedom	Mean squares	F	P	Mean squares	F	P
Between samples from suspension	2,267·98	3	755·99	1·77	>0·05	568·77	5·11	<0·001
Between dilution tubes within samples	1,710·32	4	427·56	3·21	>0·05			
Between sub-samples within dilution tubes	1,064·80	8	133·10	1·54	>0·05	111·34	—	—
Between films within subsamples	1,383·05	16	86·44	<1·0	>0·05			
Between traverses within films	10,912·40	96	113·67	4·15	<0·001			
Between fields within counts	14,054·20	512	27·45	—	—	—	—	—
Total	31,392·75	639	—	—	—	—	—	—

Analysis of the variance was carried out without transformation (Table 1). The individual mean squares are tested for significance by comparison with the immediately succeeding mean square. Thus the mean square for traverses, 113·67, is compared with that for fields, 27·45; this gives a variance ratio of

4·15, which is highly significant. The variation between the traverses was, therefore, significantly greater than that attributable to the variation between fields. The mean square for films, 86·44, is compared with that for traverses, 113·67, and is clearly not significant. The films did not therefore differ more than was to be expected from the variation between traverses. The same is true of subsamples. The variance ratio for dilution tubes, 3·21, is suspiciously high. A new estimate of the total variation within dilution tubes is made by pooling the sums of squares for subsamples, films and traverses (to give 13,360·25) and dividing by the pooled degrees of freedom (120). Since the mean square for samples is not significantly greater than that for dilution tubes, the sums of squares for these two items may also be pooled. We then have a new mean square for dilution tubes of 568·77 and for the remainder of 111·34, on 7 and 120 degrees of freedom respectively; the variance ratio of 5·11 is highly significant. There appeared, therefore, to be real differences between dilution tubes.

The components of variance may be estimated as follows:

$$\sigma_L^2 = 27{\cdot}45;$$

$$5\sigma_T^2 + \sigma_L^2 = 111{\cdot}34, \quad \text{therefore} \quad \sigma_T^2 = 16{\cdot}78;$$

$$80\sigma_D^2 + 5\sigma_T^2 + \sigma_L^2 = 568{\cdot}77, \quad \text{therefore} \quad \sigma_D^2 = 5{\cdot}72.$$

Here σ_T^2, for example, is the variance between the true mean counts from traverses on a single film. From these figures an estimate can be made of the fiducial limits for mean counts made with varying numbers of dilution tubes, traverses and fields. Thus, the estimated variance (V) of a mean count is given by

$$V = \frac{5{\cdot}72}{D'} + \frac{16{\cdot}78}{T'} + \frac{27{\cdot}45}{L'},$$

where D', T' and L' are the total number of dilutions, traverses and fields. From this it can be seen that, for any given number of fields counted, and any given number of fields per traverse, the variance is lowest when the number of dilutions made is maximum, and one traverse is made per dilution. A number of representative values are given in Table 2, and it can be seen that, with 40 or 60 fields counted, it is possible to obtain 5 % fiducial limits within 12–15 % of the mean without undue labour.

The experiment with *Strep. pyogenes* was carried out in an identical fashion, but the frequency distribution of the number of cocci per field was not normal (Fig. 2, curve *A*), owing to the fact that the cocci were distributed in chains or clumps comprising from 2 to 20 or more cocci, and no fewer than 89 of the 640 fields examined contained no cocci. Analysis of the untransformed counts gave a suspiciously low value for the between-films mean square. Transformation of the count to $\log_{10}$ (count + 1) did not produce any very obvious improvement in the shape of the frequency distribution curve (Fig. 2, curve *B*), but analysis gave a much more consistent set of mean squares (Table 3). The results, as set out in Table 3, conformed well with those obtained in the experiment with *Bact. coli* but the variance ratio due to differences between traverses was not significant, and the variance due to differences between fields was proportionately much larger, presumably as a result of the form of the frequency distribution. In consequence the accuracy of the counts, for a given number of fields examined, is less than with *Bact. coli* (Table 4).

Table 2. *Errors of estimation of mean counts of* Bact. coli *with different numbers of dilution tubes, etc.*

No. of counts (see text)						2 × (standard error)	$\frac{2 \times (\text{standard error}) \times 100}{\text{mean count}}$ (approximate 5% fiducial limits as percentage of mean)
L	*D*	*T*	*L′*	*D′*	*T′*		
5	1	4	20	1	4	6·72	25·2
	2	2	20	2	4	5·80	21·7
	4	1	20	4	4	5·30	19·9
5	1	8	40	1	8	5·84	21·9
	2	4	40	2	8	4·76	17·8
	4	2	40	4	8	4·10	15·4
	8	1	40	8	8	3·74	14·0
5	1	12	60	1	12	5·50	20·6
	2	6	60	2	12	4·34	16·3
	3	4	60	3	12	3·88	14·5
	4	3	60	4	12	3·62	13·6
	6	2	60	6	12	3·36	12·6
	12	1	60	12	12	3·06	11·5

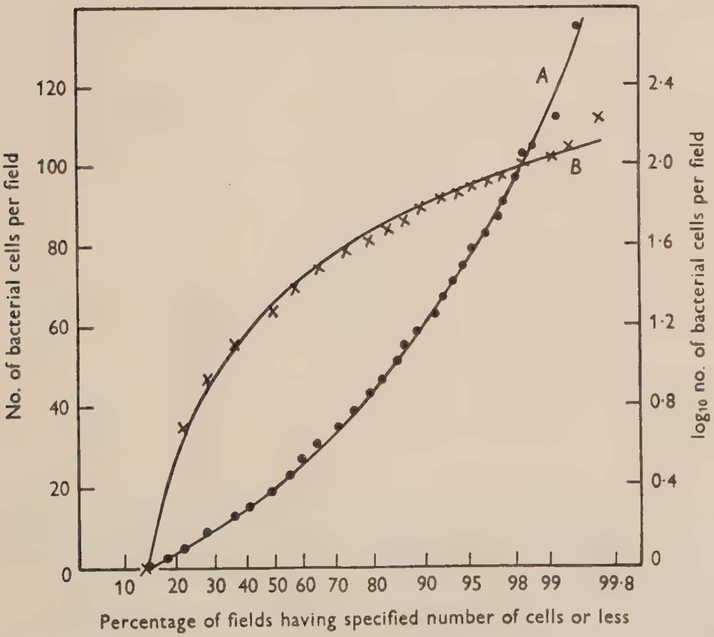

Fig. 2. Frequency distribution of numbers of *Strep. pyogenes* cells/microscope field, plotted on a probability scale. *A*, arithmetic scale for number of cells (ordinate); *B*, logarithmic scale.

However, as a further test of the validity of the analysis, the following test was made. For each film, comprising 20 fields, the number of fields with zero count was expressed as a proportion of the total 20, and this proportion transformed to angles by the method of Bliss (see Snedecor, 1946). The analysis of variance due to differences between samples, dilution tubes, subsamples and films was then carried out on the transformed variate (Table 5). The between films mean square was compared with the theoretical variance given by 820·7/20 (Armitage, personal communication), and found not to be significant. None of the individual variance ratios was significant, but the between dilution tubes mean square was suspiciously large and was further tested after pooling as shown in the table. This gave a significant value for the variance ratio due to differences between dilution tubes, which served as confirmation of the results of the analysis on the logarithms of the counts.

Table 3. *Analysis of variance of counts of* Strep. pyogenes, *transformed to logarithms*

Source of variance	Sums of squares	Degrees of freedom	Mean squares	*F*	*P*	Mean squares	*F*	*P*
Between samples from suspension	1·357	3	0·452	0·269	>0·2	1·154	3·56	<0·01
Between dilution tubes within samples	6·722	4	1·681	8·280	0·01–0·001			
Between subsamples within dilution tubes	1·620	8	0·203	0·700	>0·2	0·324	—	—
Between films within subsamples	4·640	16	0·290	0·779	>0·2			
Between traverses within films	35·701	96	0·372	1·171	0·2–0·05			
Between fields within traverses	162·684	512	0·318	—	—			
Total	212·724	639	—	—	—	—	—	—

$\sigma^2_D = 0{\cdot}010$; $\sigma^2_L = 0{\cdot}324$.

Table 4. *Errors of estimation of mean counts of* Strep. pyogenes *with different number of dilution tubes*

D′	*L′*	2 × standard error (in logarithms)	5 % fiducial limits as percentage of mean* Upper	Lower
5	10	0·372	235	42
	20	0·270	186	54
	40	0·202	159	63
	60	0·174	149	67
10	10	0·364	231	43
	20	0·262	183	55
	40	0·190	155	65
	60	0·160	145	69

* Upper limit = 100 × [antilog (2 × standard error)]; lower limit = 100 × [antilog (−2 × standard error)].

In both experiments there was a significant variance ratio due to differences between dilution tubes. Molten agar is difficult to measure accurately, and it was thought that an increase in the volume of the dilution tubes might lead to improvement. An experiment was accordingly carried out in which a single suspension of *Bact. coli* diluted with a single pipette in six 5 ml. lots of agar, and also in six 1 ml. lots, the order of making the dilutions being random. Two films were made from each dilution and two 5-field traverses carried out on each film. Analysis was carried out as before (Table 6). In this experiment the mean count from the 1 ml. dilution tubes was slightly higher than that from the 5 ml. tubes, and, although none of the variance ratios was significant there was some indication that the larger dilution volume was associated with a slightly lower variance.

Table 5. *Analysis of variance of counts of* Strep. pyogenes *transformed to angles*

Source of variance	Sums of squares	Degrees of freedom	Mean squares	*F*	Mean squares	*F*	*P*
Between samples from suspension	628·699	3	209·563	1·141	194·789	4·498	0·01–0·001
Between dilution tubes within samples	734·827	4	183·705	3·825			
Between subsamples within dilution tubes	384·169	8	48·021	1·173	43·309	—	—
Between films within sub-samples	655·252	16	40·953	<1			
Total	2402·947	31	—	—	—	—	—
Theoretical variance	—	—	41·035	—	—	—	—

Table 6. *Comparison of* 1 *and* 5 *ml. dilution tubes*

Source of variance	Sums of squares	Degrees of freedom	Mean squares	*F*	Mean squares	Degrees of freedom	*F*
		5 ml. dilution tubes					
Between dilution tubes	455·8	5	91·16	2·53	91·16	5	2·02
Between films within dilutions	216·5	6	36·08		45·03	114	—
Between traverses within films	310·8	12	25·90				
Between fields within traverses	4606·6	96	47·99				
Total	5589·7	119	—		—	—	—
		1 ml. dilution tubes					
Between dilution tubes	728·8	5	145·76	2·13	145·76	5	2·78
Between films within dilutions	410·3	6	68·38		52·49	114	—
Between traverses within films	292·2	12	24·35				
Between fields within traverses	5281·0	96	55·01				
Total	6712·3	119	—		—	—	—

DISCUSSION

It is apparent that the two-stage method of carrying out counts of the total number of bacteria in a suspension, by the use of cast agar films, can give a degree of precision of the same order as that given by the direct counting of the bacteria in a Helber chamber, although no thorough analyses of the latter method appear to have been published.

The advantages of the two-stage method of counting are the fact that haemocytometer chambers, which are readily available, can be used, and that the time-consuming process of counting the cells can be postponed to the most convenient occasion if, for some reason, it is thought undesirable to hold the suspensions meanwhile. No change has been observed in mounted or unmounted films kept for several days on the bench. The disadvantage of the method is that the total time taken over the procedure is greater than in a direct counting method, and that practice is required before the several stages can be carried out easily.

It is clear from the analyses that the greater part of the variation between counts is accounted for by the variation between successive fields counted. There is of course a limit, set by the nature of a random distribution of cells across the film, below which this variation cannot be reduced. The fact that, in the experiment with *Bact. coli*, σ^2_L had the value of 27·45 compared with that of 26·69, which would be the estimate of the variance of a Poisson distribution with the same mean, suggests that this limit is approached. There is, nevertheless, a moderate additional variation produced by differences between traverses, each of five fields, on the film. It has been observed that, in some films, the count is higher at the edge than in the centre. This may well account for some of the between-traverses variation, and it was to reduce the risk of errors from this source that the practice of making the traverses along one axis of the film was adopted. The only other significant source of variation was that due to different dilution tubes and the indications are that this was truly a difference between the tubes and not between the pipettes used for measuring the primary samples, although it is difficult to be certain since there are only 3 degrees of freedom available for measuring the latter variation. It is probable that the effect of the dilution tubes could be reduced by using volumes larger than 1·0 ml., although with the larger volume it would be more difficult to ensure mixing.

The number of dilution tubes, films and traverses that would be needed in practice must of course depend on the accuracy required, and also on the density of the cells in the preparation. It is suggested that, with a suspension consisting largely of single cells, and of such density as to give a count per field of the same order of magnitude as in the present work, a reasonable compromise might be to use four dilution tubes, make one film from each tube and perform two traverses, each of five fields, on each film. This amounts to counting 40 fields or, with a density of 25 cells per field, 1000 cells. In the ordinary way it will probably be sufficient to estimate the standard error of an individual count from the variance of the mean counts from the four dilution tubes.

I am very grateful to Dr P. Armitage, of the Medical Research Council's Statistical Research Unit, for guidance on the methods of statistical analysis and for many helpful comments.

REFERENCES

HELBER, E. (1904). Über die Zählung der Blutplättchen im Blute des Menschen und ihr Verhalten bei pathologischen Zuständen. *Dtsch. Arch. klin. Med.* 81, 317.

JONES, P. C. T. & MOLLISON, J. E. (1948). A technique for the quantitative estimation of soil micro-organisms. *J. gen. Microbiol.* **2**, 54.

SNEDECOR, G. W. (1946). *Statistical Methods*, 4th ed. Ames, Iowa: Iowa State College Press.

Topley and Wilson's Principles of Bacteriology and Immunity (1946), 3rd ed. Revised by WILSON, G. S. & MILES, A. A. London: Edward Arnold and Co.

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Harper, G. J. & Morton, J. D. (1952). *J. gen. Microbiol.* 7, 98–106

Bacillus subtilis Spores labelled with Radiophosphorus

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SUMMARY: *Bacillus subtilis* was grown in surface culture on media containing about 0·6 mC./ml. of ^{32}P as phosphate, and harvested after 2 days. The spore suspension was heated at 60° for 1½ hr. and washed by centrifuging and resuspending in water six times. The fresh suspension contained about 2×10^9 spores/ml., of which 20 % were viable. The radioactivity was about 0·05 mC./ml. of which more than 95 % was carried by the spores. The radioactivity was sufficient for 10^3 spores to be readily detected and assessed by conventional Geiger-Müller counting methods. These highly radioactive suspensions are particularly useful in studying the respiratory retention of bacterial aerosols.

A number of investigations have been reported in which micro-organisms have been labelled with radio-isotopes by growing them in media containing the isotope in a chemical form suitable for assimilation. In those experiments the level of radioactivity in the media was generally low, of the order of a few micro-curies/(mC.) ml. It seemed likely that the concentration of radioactive material could be considerably increased without serious loss in the yield of labelled micro-organisms. Because of the probable usefulness, in various studies, of micro-organisms labelled to the highest practical degree of radioactivity, we decided to investigate the use of media of much higher activity than any previously reported.

Bacillus subtilis was chosen for this work because of the relatively high resistance of spores to radiation damage and because *B. subtilis* is a robust and easily handled organism. We used a locally selected strain that has the general characteristics of *B. subtilis* and forms spores freely at 37° in 2 days. Radiophosphorus (^{32}P) was an obvious choice: phosphorus is readily assimilated from simple inorganic phosphates and forms 2–3 % by weight of the spore, and the isotope emits hard β-rays that are easily counted.

The purpose of this paper is to describe the preliminary work, to give the method finally selected, and to note some properties and uses of the highly radioactive spore suspensions obtained.

Radiation dosage

The biological effects of heavy radiation dosages on spores and on resting suspensions of vegetative organisms have been extensively studied (Lea, 1947), but nothing appears to have been previously reported on growing organisms in comparable radiation fluxes. It is of interest to calculate the β-radiation dosages affecting our *B. subtilis* during growth and in subsequent storage.

Note on units of dosage

The roentgen (r.) is the unit of radiological dose and is defined in terms of the ionizing power of X- or γ-radiation. For radiation other than X- or γ-radiation, the roentgen-equivalent-physical (rep.) is used, and is defined as the radiological dose which produces the same energy absorption in tissue as 1 r. of X-rays or γ-rays. The millirep (m.rep.) is a useful smaller unit.

The organisms were grown on solid medium of activity up to about 600 mC./l., the incubation period being about 45 hr., and the harvested spore suspensions were stored for various periods.

Surface dosage on active medium

We are indebted to A. C. Chamberlain, Atomic Energy Research Establishment, Harwell, for a modification of the treatment of this case given by Parker (1948).

The dosage on the surface of a radioactive material (so long as it is composed of light elements) is half what it is in the middle. Parker gives for a submerged object

Dosage rate $= 2{\cdot}6\ CE/\rho$ m.rep./hr.,

where C = activity in μC./l.; for our most active media this is about 6×10^5 μC.: E = average β-ray energy; for ^{32}P this is about 0·7 Mev. (Million electron volts): ρ = density; for our media this is 1·06 g./c.c. The dosage rate is therefore

$\frac{1}{2}\frac{2{\cdot}6 \times 6 \times 10^5 \times 0{\cdot}7}{1{\cdot}06} = 5{\cdot}15 \times 10^5$ m.rep./hr., or 515 rep./hr., and the total dosage in the 45 hr. incubation period is $515 \times 45 = 2{\cdot}3 \times 10^4$ rep.

Self-irradiation in the spore suspension

The freshly harvested spore suspensions from medium of activity about 600 mC./l. give about 25×10^7 counts/min. from 20 ml. Since the Geiger-Müller (GM) counter is about 10 % efficient, the number of disintegrations/min./ml. is

$$\frac{25 \times 10^7}{20} \times 10 = 1{\cdot}25 \times 10^8$$

1 μC. $= 2{\cdot}2 \times 10^6$ disintegrations/min. and the activity of the suspension is therefore

$$\frac{1{\cdot}25 \times 10^8}{2{\cdot}2 \times 10^6} = 57\ \mu\text{C./ml.,} \quad \text{or} \quad 57 \times 10^3\ \mu\text{C./l.}$$

Owing to radioactive decay, the mean activity over a period of 14 days will be about 42×10^3 μC./l. Using the formula given above, the dosage rate over this period will be $2{\cdot}6 \times 42 \times 10^3 \times 0{\cdot}7 = 7{\cdot}6 \times 10^4$ m.rep./hr. (The density is taken as 1·0 g./c.c.) The total dosage is therefore $7{\cdot}6 \times 10^4 \times 10 \times 24 = 2{\cdot}6 \times 10^7$ m.rep. or $2{\cdot}6 \times 10^4$ rep. in 14 days. This is probably an underestimate, since the ^{32}P is mostly contained within the spores which settle out and increase the concentration of activity in their vicinity. Summarizing these calculations we may say that the organisms receive a dosage of about $2{\cdot}3 \times 10^4$ rep. during the incubation period and probably more than 3×10^4 rep. during 14 days storage.

The literature gives median lethal dosages (M.L.D.) of, for example, 4×10^3 rep. (β-radiation) for *Bacterium coli*, and $1{\cdot}1 \times 10^5$ rep. for *B. mesentericus* spores (Lea, Haines & Bretscher, 1941). If the *B. subtilis* used here be assumed to spend 1 day of the incubation period in vegetative multiplication, it probably received two or three M.L.D. during this period; this is in agreement with the low yield of spores: namely, about 40 % of that on inactive medium (see below). It was also found that only 20 % of the harvested spores were viable as shown by the ratio of viable to total (optical density) count (see Table 3) When the spores were examined by a technique developed by Pearce & Powell (1951), this was confirmed, and the further interesting observation was made that a number of the spores that were non-viable in the sense of being unable to form macroscopic colonies did, however, germinate and might achieve one or two divisions before growth ceased and lysis took place. This suggests the formation of 'lethal mutants' as described by Lea (1947). The decrease in viable count due to self-irradiation is shown in Table 1, which compares suspensions grown on media of various degrees of radioactivity.

Table 1. *Influence of storage after harvesting on viable counts of* B. subtilis *spore suspensions grown on CCY agar containing various amounts of* ^{32}P: *viable counts are expressed as percentages of original counts on day of harvesting*

Suspension no.	Radioactivity of medium (mC./l.)	Days storage									
		1	2	3	6	8	10	15	20	30	49
12	605	98	88	69	65	54	43	30	22	7	4
14	60·5	99	106	108	102	102	102	103	—	—	—
15	0	109	103	111	103	108	107	108	109	102	97

Safety precautions

The precautions taken in handling the radioactive materials followed established practice, but a short description may be helpful to workers unfamiliar with this field. Surgical gloves and gowns were used for all operations with highly radioactive material. A consignment of, say, 120 mC. of $Na_2H^{32}PO_4$ in 10 ml. of water was received in an ampoule, which was opened by blow-pipe flame. The contents were transferred to a sterile test-tube by means of a Pasteur pipette and rubber bulb, and 20 mC. lots were distributed into bottles containing 30 ml. of medium by means of a graduated pipette with rubber bulb. Reliance was placed on rapid handling to reduce radiation dosage, and film badges on the wrist showed that the dosage received was below the limit of tolerance.

The glass culture bottles used were only 2–3 mm. thick, and metal shields were therefore used during incubation. Subsequent operations such as harvesting and washing spores were done with bulb-operated pipettes, and reliance was again placed on rapid handling rather than shielding to avoid radiation injury. The bench top was protected by metal trays lined with filter-paper. Gloves, gowns and bench were monitored after the operation, not only for reasons of health but also to detect contamination which might affect experimental material. We have found that normal bacteriological techniques are well suited to this kind of work. Bottles containing expended media, and glassware used in handling strong suspensions, were placed in a metal container and stored for several months until the activity had decayed to a tolerable level. Other glassware was rinsed before being returned for normal washing up.

Preliminary observations

Medium. CCY agar (Gladstone & Fildes, 1940) gave the best yield of spores; experiments with defined media, undertaken to give more controllable total phosphorus content, were abandoned when it was found that the yields of spores were always much lower than on CCY. The normal method of preparation of the CCY agar was modified by omitting the glycerophosphate, so as to diminish the content of non-radioactive phosphorus. This modification did not decrease the spore yield.

Agar. Shredded agar (British Drug Houses Ltd., Kobe No. 1) gave the best yield of spores. 'Oxoid' powdered agar gave an increased uptake of ^{31}P and ^{32}P/spore, but the total yield of spores was so much lower that the utilization of the ^{32}P was less efficient than with the shredded agar (Table 2).

Table 2. *Influence of type of agar on yield of spores when* B. subtilis *was grown on normal and radioactive CCY*

Suspension no.	Radioactivity (mC./l.)	Agar	Spores/bottle ($\times 10^{-10}$)		Viable (%)	GM count/bottle ($\times 10^{-7}$)	GM count/10^5 spores		^{32}P utilized (%)
			Total	Viable			Total	Viable	
29	0	Shr.	9·0	8·0	89	—	—	—	—
18	0	Ox.	1·8	1·6	89	—	—	—	—
23	0	Ox.	3·0	1·9	63	—	—	—	—
26	0	Ox.	2·52	2·06	82	—	—	—	—
32	0	Ox.	2·16	1·48	69	—	—	—	—
30	59·5	Shr.	8·4	3·6	43	3·96	47	108	7·7
31	59·5	Ox.	1·8	0·804	45	2·38	132	296	4·6
19	60·2	Ox.	2·4	0·796	33	2·57	107	323	4·8
12	605	Shr.	3·6	0·816	23	32·7	910	4000	6·3
16	635	Ox.	1·8	0·091	5	7·12	398	7720	1·3

Films of suspensions from shredded agar showed complete spore formation, but those from 'Oxoid' agar only about 30 %. Shr. = shredded agar; Ox. = 'Oxoid' powdered agar.

Method of cultivation. Surface culture on 30 ml. solid medium in flat 8 oz. bottles was preferred. The bottles facilitated handling of the highly radioactive medium, and the radiation dosage in surface culture is one-half that in liquid medium.

Radiation shielding. The radiation dosage during the incubation period was of the order of the M.L.D. for vegetative organisms. If this dosage could be diminished without affecting other conditions, the number of organisms growing and surviving to form spores should be increased. It was thought that a layer of unglazed porcelain lying on the upper surface of the medium would substantially reduce the β-radiation without interfering with the transport of nutrient material to organisms inoculated on the upper surface of the porcelain. It was found, however, that the yield of viable spores obtained when using inactive media was so much diminished, in a variety of test assemblies, that the method was not worth pursuing.

Cellophan-covered medium. The initial water soluble activity of the harvested spore suspensions is high, necessitating tedious repeated washings. It was thought that growth on cellophan covering the agar might decrease this activity by enabling the spores to be stripped cleanly from the medium, but it was found that the yield of spores was halved without any advantage being gained.

Quantity of medium. Volumes of 30 ml. medium/8 oz. bottle were used in the first experiments. It was thought that this might be an unnecessarily large volume, so that the spore yield was limited by surface area rather than by available nutrients; some economy in radiophosphorus might therefore be obtained by use of less medium. Experiments with inactive medium showed, however, that decrease of the volume of medium below 30 ml./bottle gave decreased spore yield/ml. medium.

Phosphorus content of medium. The ^{32}P was added as Na_2HPO_4, a readily utilizable form of phosphorus. If the phosphorus-31 in the medium were less readily available, the ^{32}P would be taken up preferentially. If the ^{31}P were present in as readily available a form as the ^{32}P, then the proportion of the radiophosphorus taken up by the spores would be the same as the proportion of inactive phosphorus taken up. Some experimental results bearing on this are given.

(i) A bottle of CCY agar containing 4·77 mg. total phosphorus gave a spore suspension that contained, after washing, 0·522 mg. phosphorus. The uptake of phosphorus was therefore $(0{\cdot}522/4{\cdot}77) \times 100 = 10{\cdot}9\ \%$ of that present initially in the medium. The proportion of ^{32}P taken up from media of low activity was about 15 % (see Table 3). This indicates that the uptake of the two isotopes was similar, but that part of the ^{31}P was probably in a less readily utilizable form. Inorganic phosphates are more readily utilizable than more complex organic compounds.

(ii) When glycerophosphate was added to CCY medium, the uptake of ^{32}P was much decreased, although the yield of spores was unchanged. This suggested at first that ^{31}P as glycerophosphate was more readily available than ^{32}P as phosphate, but when the addition of ^{32}P as glycerophosphate was tried the uptake was again lower than in the control suspension. It is probable that the decreased uptake of ^{32}P was due to there being a large excess of available phosphorus in the medium after the addition of glycerophosphate. The phosphorus in glycerophosphate is probably no more readily assimilated than in inorganic phosphate. The decreased uptake also found when radioactive glycerophosphate was added was probably due to the presence of inactive 'carrier' glycerophosphate, present after synthesis of the active material, and of which 6 mg. was added to each bottle.

Preferred method of preparation of labelled spore suspensions

The method selected for routine preparation of radioactive suspensions is as follows:

Thirty ml. CCY agar (without glycerophosphate) are melted in an 8 oz. 'medical flat' (medicine bottle $1\frac{1}{2}$ by $2\frac{1}{2}$ by $5\frac{1}{4}$ in., excluding neck) and about 20 mC. of $Na_2H^{32}PO_4$ in 1–2 ml. water added. The agar is allowed to set with the bottle lying on its side and the surface is then inoculated with 1·25 ml.

B. subtilis spore suspension containing about 2×10^8 spores. The bottle is incubated in this position at 37°, in a metal shield to screen β-radiation, for about 45 hr. The growth is washed off with 20 ml. distilled water into a 1 oz. screw cap bottle, shaken by hand with glass beads to break up clumps, and heated at 60° for 1½ hr. Water soluble ^{32}P is removed from the suspension by centrifuging and resuspending in water six times, after which the activity of the sixth supernatant fluid is less than 5 % of the activity of the whole suspension.

Table 3. B. subtilis *spore suspensions grown on CCY agar containing* ^{32}P

Suspension no.	Radio-activity (mC./l.)	Spores/bottle ($\times 10^{-10}$)		Viable (%)	GM count/bottle ($\times 10^{-7}$)	GM count/10^5 spores		^{32}P utilized (%)
		Total	Viable			Total	Viable	
15	0	9·6	9·4	98	—	—	—	—
29	0	9·0	8·0	89	—	—	—	—
35	0	9·0	7·82	87	—	—	—	—
2	40·0	—	6·72	—	5·06	—	75	14·6
9	42·0	4·8	4·48	93	4·59	96	102	15·2
30	59·5	8·4	3·6	43	3·96	47	108	7·7
38	58·1	6·4	4·36	68	7·1	111	163	14·3
14	60·5	7·8	4·52	58	5·8	74	128	11·1
33	250	4·8	2·4	50	17·9	374	747	8·4
3	320	—	1·64	—	14·9	—	910	5·4
34	571	5·0	1·06	21	26·1	522	2460	5·4
37	581	4·0	1·03	26	25·5	638	2460	5·1
28	595	4·8	0·96	20	26·2	546	2730	5·1
4	638	—	1·08	—	21·8	—	2020	4·0
12	605	3·6	0·816	23	32·7	910	4000	6·3

The activity of the medium is based on the activity of the radioactive phosphate solution as given by the A.E.R.E., Harwell. For convenience in subsequent calculations, both this and the GM count of the suspension have been corrected for decay to one day after inoculation of the bottle: that is, half way through the incubation period. All counts are in pulses per minute and were taken in equipment of about 10 % counting efficiency.

The total number of spores/ml. is estimated from optical density measurements. We found that these measurements were reasonably reliable both by determinations on fully viable non-radioactive suspensions and by microscopical examination which showed no abnormal proportion of debris. The viable count was made by the plate-dropping technique of Miles & Misra (1938).

Radioactive counts were done either with known volumes dried on aluminium planchettes and presented to an end-window counter (GM 2 or GM 4; General Electric Co.), or with M 6 liquid counters (Veall, 1948; 20th Century Electronics Ltd.). The end-window counter was particularly useful for checking the decreases of activity in the supernatants during washing. The liquid counter was used for determinations in connexion with the experimental use of active suspensions, so that direct comparisons might be made between suspension activity and experimental results obtained with liquid preparations.

Table 3 gives details of suspensions prepared by the routine method at various levels of radioactivity, including control suspensions prepared on non-radioactive medium.

The following points are worth comment: (*a*) the *total* yield of spores was progressively decreased with increasing radioactivity of medium to about 40 % of the yield on non-radioactive medium; (*b*) the yield of *viable* spores decreased from 100 % of the total yield on inactive medium to 20 % on the most active medium; (*c*) from the most active medium, 10^3 spores give about 7 counts/min. and, therefore, using an M 6 tube with background count 12 counts/min., the probable error on a 20 min. count is less than 5 %; (*d*) the proportion of ^{32}P found in the suspension decreased with increased activity of medium. The decrease appears to be due to the smaller number of spores formed.

Properties of the suspensions

If measurements of radioactivity were to be used as a reliable quantitative index of the presence of labelled spores, it was evidently necessary to be sure that substantially all the ^{32}P in the suspensions was firmly bound to the spores. Experimental results given below show that there was always some radioactivity in the suspending fluid and that the amount increased on standing. However, this amount of activity may frequently be neglected, particularly when the suspension is washed just before use.

Table 4. *Supernatant activity as percentage of total activity of suspension after final wash*

No. of washings	Suspension no.			
	17	19	34	37
1	390	360	—	290
2	46	39	26	21
3	11·3	8·1	5·1	4·6
4	5·4	5·2	5·7	2·0
5	3·1	3·0	6·8	—
6	3·1	2·8	5·4	1·5
7	5·1	1·4	3·7	—
8	4·5	—	3·4	—
9	2·0	—	—	—

Water soluble radioactivity. When the freshly harvested suspensions were repeatedly centrifuged with fresh lots of water, successive washings showed progressively less activity (Table 4). When suspensions were allowed to stand in the refrigerator the amount of activity in the supernatant increased. Repeated centrifuging and resuspension in the same fluid gave with one suspension a supernatant having 5 % of the total suspension activity after 1 day, rising to 22 % after 10 days. It seems possible that this increase was caused by slow diffusion out of dead spores and debris.

Phosphorus exchange. Inactive *B. subtilis* spores suspended in $Na_2H^{32}PO_4$ solution of activity 0·03 mC./ml. for 41 hr. at 4° did not retain significant activity. Successive washings showed very rapid decrease in the activity of the suspension, which was at each stage approximately equal to the activity of the supernatant fluid after centrifuging. This suggests that the ^{32}P in the labelled suspensions is actually incorporated in the cell substance. This was confirmed by experiments indicating that ^{32}P did not exchange with ^{31}P when the cells were suspended in phosphate buffer, and also by the similarity in uptake of ^{32}P and of total phosphorus during growth. Surface active agents are known to increase cell surface permeability in certain circumstances. Experiments showed that a surface active agent did not significantly affect the loss of ^{32}P from labelled spores. Radioactive spores were suspended in water and in 0·7 % 'Tergitol' (a branched long chain sodium sulphonate; General Metallurgical and Chemical Corporation, Holborn, London), and stored at 4° for 46 hr. The activity of the whole suspension and of the supernatant after centrifuging were then measured. In one experiment the supernatant activity was 37 % of the whole suspension activity in the water suspension, and 56 % in the Tergitol suspension; a repeat experiment gave 42 % in water and 41 % in Tergitol. It was concluded that the Tergitol had no significant effect. This is in agreement with other observations which show that heat stable spores are usually indifferent to substances which attack or modify the vegetative cell wall.

Applications

These highly radioactive suspensions are particularly useful in studying the respiratory retention of bacterial aerosols. It is easy to detect, and to assess with sufficient accuracy, numbers of the order of 10^3–10^4 spores, so that experimental animals do not have to be exposed to very concentrated aerosols for long periods. We have published a preliminary account of such work (Buckland, Harper & Morton, 1950); similar work has been reported by Goldberg & Leif (1950), who used avirulent *Pasteurella pestis* labelled with ^{32}P.

In a previous communication (Buckland *et al.*, 1950) reference was made to the loss of viable organisms frequently observed when dilute suspensions of normal spores are bubbled or shaken, or even allowed to stand at room temperature or in the refrigerator. This loss has usually been ascribed to aggregation or to actual loss of viability; aggregation has been directly observed. However, we have found corresponding decreases of radioactive count, and found the spores, irrespective of aggregation, were adsorbed on the wall of the vessel. This is a factor of considerable importance in any investigation of chemical and physical influences on viability, and may cause doubt as to the validity of some reported results. The use of labelled organisms greatly facilitates the study of apparent losses of viable organisms due to adsorption on vessel walls.

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REFERENCES

BUCKLAND, F. E., HARPER, G. J. & MORTON, J. D. (1950). Use of spores labelled with radiophosphorus in the study of the respiratory retention of aersols. *Nature, Lond.* **166**, 354.

GLADSTONE, G. P. & FILDES, P. (1940). A simple culture medium for general use without meat extract or peptone. *Brit. J. exp. Path.* **21**, 161.

GOLDBERG, L. J. & LEIF, W. R. (1950). The use of a radioactive isotope in determining the retention and initial distribution of airborne bacteria in the mouse. *Science*, **112**, 299.

LEA, D. E., HAINES, R. B. & BRETSCHER, E. (1941). The bactericidal action of X-rays, neutrons, and radioactive radiations. *J. Hyg., Camb.* **41**, 1.

LEA, D. E. (1947). *Actions of Radiations on Living Cells.* Cambridge University Press.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

PARKER, H. M. (1948). In *Advances in Biological and Medical Physics*, **1**, 275–8. New York: Academic Press Inc.

PEARCE, T. W. & POWELL, E. O. (1951). New techniques for the study of growing micro-organisms. *J. gen. Microbiol.* **5**, 91.

VEALL, N. (1948). A Geiger-Müller counter for measuring the beta-ray activity of liquids. *Brit. J. Radiol.* **21**, 347.

(*Received* 6 *February* 1952)

Jackson, F. L. & Copping, Alice M. (1952). *J. gen. Microbiol.* 7, 107–116

Some Aspects of the Growth of *Proteus vulgaris* with Limiting Concentrations of Nicotinic Acid

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SUMMARY: When *Proteus vulgaris* is grown in a simple defined medium with limiting concentrations of nicotinic acid but all other constituents in excess, growth as measured by dry-weight determinations continues for several hours after the disappearance of all detectable nicotinic acid from the medium. A linear phase of protoplasmic synthesis and of oxygen uptake follows this disappearance and lasts for 2·5–3 hr., while the amount of recoverable nicotinic acid in the cells remains constant. It is suggested that, during the period of linear growth, the rate of protoplasmic synthesis is determined by the presence within the total cell population of a more or less constant amount of nicotinic acid or its active derivatives.

When an organism grows under specified conditions, protoplasmic synthesis follows a characteristic course, and the more precisely the conditions of both inoculum and medium can be controlled, the more reproducible will be the results of growth experiments. For quantitative growth studies and for some investigations of metabolism, it may be of particular value if growth is limited by the concentration of a known single factor. Some microbiological assays depend on measurement of the growth response of an organism under conditions in which the concentration of the substance being assayed is the sole limiting factor. The relation between utilization of the factor and the course of protoplasmic synthesis is therefore of interest, and may be expected to vary in different cases. If the factor is inactivated quickly after assimilation, growth may soon cease when an external supply is no longer available. Where inactivation of the factor is less rapid, a stage will be reached when no external supply remains, but the cells contain a certain amount of the factor, or of its derivatives, in a functional state. In such a case, particularly if the factor is to function as a coenzyme for a limited number of enzyme reactions, cell substances whose synthesis is not directly dependent on the utilization of the factor as a 'building block' might still be formed. In other words, in certain cases, although no external supply of the growth factor remains, cell growth might be expected to continue for a considerable period.

Nicotinic acid is a growth factor for certain organisms and is known to be required for the formation of the pyridine nucleotide coenzymes. A culture of an organism unable to synthesize nicotinic acid might continue to grow after all the growth factor had been assimilated if active nicotinic acid derivatives were available in the cells. If, for a certain period, the activity of these derivatives was more or less constant, the rate of protoplasmic synthesis might become constant, being determined by the activity of those enzyme systems dependent on the supply of nicotinic acid. Under these circumstances, a linear growth phase would occur, and would continue until there was significant inactivation of the nicotinic acid derivatives, or until some other change in conditions limited growth.

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To test the possibilities outlined, we have investigated the growth of a strain of *Proteus vulgaris* in a simple defined medium under conditions in which nicotinic acid concentration was the sole factor limiting growth.

METHODS

Organism. A strain of *P. vulgaris*, C.N. 2433, obtained from Mr H. Proom of the Wellcome Research Laboratories, Beckenham, was selected from a number of strains tested and was used throughout. It was maintained on meat-digest agar slopes. Before use, subcultures were made in defined medium containing a limiting quantity of 0·03μg. nicotinic acid/ml. Inocula from such cultures were twice washed with nicotinic acid-free medium before use. Glass-distilled water was used for all solutions and washings unless otherwise stated.

Medium. Preliminary experiments were made with an ammonium lactate medium based on that of Fildes (1938). The effects of varying the concentrations of lactate and ammonium salts, with different concentrations of nicotinic acid, were investigated and a medium was devised which allowed nicotinic acid concentration to be made the sole limiting factor for growth, all other constituents being present in excess. The final composition of the medium adopted was: M-KH_2PO_4, 15 ml.; M-NH_4Cl, 18 ml.; 0·2 M-$(NH_4)_2SO_4$, 36 ml.; 0·5 M-lactate, 90 ml.; 0·0167 M-$MgSO_4$, 4·5 ml.; 0·002 M-$(NH_4)_2SO_4 . FeSO_4 . 6H_2O$, 11·25 ml.; nicotinic acid solution (concentration varied), 4·5 ml.; distilled water to 450 ml.; pH adjusted to 7·2.

The lactate solution was prepared from analytical quality lactic acid; 4·5 g. of the acid were diluted with 20 ml. water, and the pH of the boiling solution adjusted to 7·0 with N-NaOH. The volume was made up to 100 ml.

The ferrous ammonium sulphate solution was made up in 0·02 N-HCl and sterilized by Seitz filtration. The lactate solution was autoclaved at a pressure of 10 lb. for 10 min. and the mixture of inorganic salts was autoclaved as a separate solution.

Stock nicotinic acid solution (0·001 M) was sterilized by Seitz filtration.

Cultures were grown in Erlenmeyer flasks at 36° and were shaken continuously during growth at a rate of 92 complete oscillations/min. and an amplitude of 5 in. This ensured adequate aeration, and increased rate of shaking did not increase the growth rate or the final yield. Quantities of culture medium varied from 10 ml. in a 50 ml. flask to 300 ml. in a 3 l. flask.

Opacity measurements. Opacities were measured by means of a Spekker absorptiometer using filters H 508, with defined medium in the blank cell. The dry weight-opacity relationship was determined for organisms harvested at the end of the exponential phase of growth in defined medium and for organisms harvested after cessation of growth, and was found to be the same in each case The dry weight (at 110°) was determined on suitable samples of the thrice-washed original heavy suspension.

Total counts. A Helber counting chamber was used, with low-power dark-ground illumination and a × 25 compensating eye-piece, following the method described by Wilson (1922). Samples (1 ml.) of culture were transferred to 1 ml. phenol saline, and any further dilution necessary was made with water.

Viable counts. The method of Miles & Misra (1938) was used. Plates were prepared with meat infusion agar (Wright, 1933), containing sodium lactate (10 mg./ml.), and boric acid (1 mg./ml.) was added to inhibit swarming (Sykes & Reed, 1949).

Nitrogen determinations. Cells from 10 ml. samples of culture were washed three times with water at 4°. Their nitrogen content was determined by a modification of the Kjeldahl method described by Miller & Houghton (1945), a Markham distillation apparatus being used.

Oxygen uptake. Samples (2·6 ml.) of culture which had reached measurable opacity were transferred to Warburg manometer vessels with 0·2 ml. KOH and a filter-paper strip in the centre well and air in the gas phase. They were shaken at 120 oscillations/min. in a bath at 36°. Growth curves for these experiments were prepared from culture samples shaken in 50 ml. flasks in the same bath.

Nicotinic acid assays. The method of Barton-Wright (1946) was used. For each estimation, cells from 200 ml. samples of culture were collected by centrifugation at 12,500 r.p.m. in an angle centrifuge and washed twice at 4° with nicotinic acid free medium before acid extraction. Omission of the washing of cells harvested from cultures with no detectable nicotinic acid in the supernatant fluid did not affect the result.

RESULTS

Growth in the medium

The term 'growth' is here used to denote increase of the total mass of bacterial protoplasm in the culture, as estimated by dry-weight determinations based on opacity readings. Curves of total numbers and of cell nitrogen will be referred to as such, and will not be called growth curves.

In a series of cultures in which nicotinic acid concentration ranged from 0 to 0·05μg./ml., the curve relating dry wt./ml. after 48 hr. incubation to initial nicotinic acid concentration was linear; concentrations of nicotinic acid above 0·05μg./ml. gave progressively less increase, until a maximum dry weight was reached at about 0·10μg./ml. Results after 72 hr. incubation were the same as at 48 hr. and variations from batch to batch of medium were slight. In the experiments to be described, nicotinic acid concentrations corresponding to values on the linear part of the standard curve were used, and varied in different experiments from 0·02 to 0·03μg./ml. With a concentration of 0·03μg./ml. the pH of the medium at the end of growth had risen to 7·6.

The inoculum was about 2×10^4 viable cells/ml. medium and a measurable opacity developed after 18–22 hr. Total counts were possible before there was sufficient growth to allow accurate dry-weight estimations. The time at which observations were begun is shown as zero time in the figures, since in this investigation the later phases of growth are of particular interest, and slight variations in the time interval between inoculation and development of a measurable opacity were not associated with significant changes in the later growth phases. Increasing the inoculum tenfold shortened the lag-phase but did not affect the final cell mass or the form of the later phases of the growth curve.

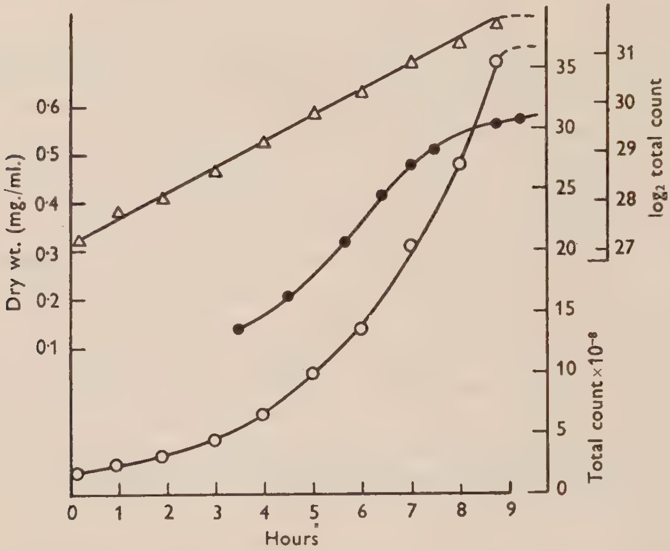

Fig. 1. **Curves of growth and of increase in total numbers in a culture containing initially 0·03 µg. nicotinic acid/ml. ●—●, dry wt.; ○—○, total numbers; △—△, $\log_2$ total numbers.**

Growth curves, total numbers and cell nitrogen

Curves constructed from estimations of dry weight and of total numbers are shown in Fig. 1. The culture contained initially 0·03 µg. nicotinic acid/ml. The total numbers rose exponentially throughout the period of observation. The dry-weight curve was found to show initially an exponential phase after readings became possible, and the terminal portion of this phase is seen in Fig. 1. The exponential phase of synthesis was followed by a phase which was linear for 2·5–3 hr. and then growth was rapidly retarded. The total numbers did not increase further after the dry weight had become stationary.

During the period of measurement the dry weight increased only by a factor of 4·2 while total count increased seven-fold. This implies a reduction in size of the units counted, and measurements of photographs showed that the reduction is mainly in length of the organisms.

The curve relating cell nitrogen to incubation time showed the same characteristics as the dry-weight curve.

Disappearance of nicotinic acid from the medium

A growth experiment was carried out as in the last section with 150 ml. medium. Samples (10 ml.) were collected at intervals, cooled and centrifuged at 4°. The supernatant fluids were transferred to 50 ml. flasks, re-inoculated and re-incubated. The opacities which developed after 48 hr. were measured, and by comparison with a standard curve constructed for the same batches of nicotinic acid and medium at the same time, gave an approximate value for the residual nicotinic acid in the samples. More nicotinic acid was then added to the flasks and incubation was continued. This demonstrated that with adequate nicotinic acid all flasks would show growth to the expected level for the amount added, and that it was due only to lack of nicotinic acid that growth was slight or absent in the samples of supernatant fluid.

The cells from the same samples were washed three times, at 4° with nicotinic acid-free medium, and were then suspended in nicotinic acid-free medium to a density of about 0·04 mg. dry wt./ml. Samples of 10 ml. were transferred to 50 ml. Erlenmeyer flasks and were incubated for 48 hr. at which time the opacities were read. Nicotinic acid was then added and incubation was continued to show that those samples in which little or no growth had occurred, would grow if supplied with nicotinic acid.

The results of these experiments (Fig. 2) showed that a considerable increase in dry weight occurred after the disappearance of all detectable nicotinic acid from the medium. The growth curve shows that, following the disappearance of nicotinic acid from the medium, there was, as usual, a phase of more or less linear growth which lasted for 2·5–3 hr., and that this phase was followed by rapid retardation of growth. During this time, the ability of washed cells to grow on transference to nicotinic acid-free medium progressively decreased.

The effect of adding more nicotinic acid to a culture which was initially provided with a limiting concentration of 0·02 μg. nicotinic acid/ml. is shown in Fig. 3. After growth had become measurable the culture was divided into three 50 ml. portions, each in a 250 ml. flask. Addition of extra nicotinic acid before the end of exponential growth (A_1) did not produce an immediate growth response. By the time the sample to which no extra nicotinic acid was added (A_3) was in the 'linear' phase, A_1 was still growing exponentially. Addition of more nicotinic acid to the third sample (A_2) during the linear phase of growth was followed by a more rapid response.

Nicotinic acid content of cells

The amount of nicotinic acid recoverable from the cells in a given volume of culture was found to remain constant during and after the phase of linear growth (Table 1). The quantity of nicotinic acid which could be accounted for by the methods used was only 50–60 % of the nicotinic acid originally present in the medium and the fate of the rest of the nicotinic acid is not known. To find whether nicotinic acid was being destroyed during the extraction process, a known amount of nicotinic acid was added to a cell suspension just before

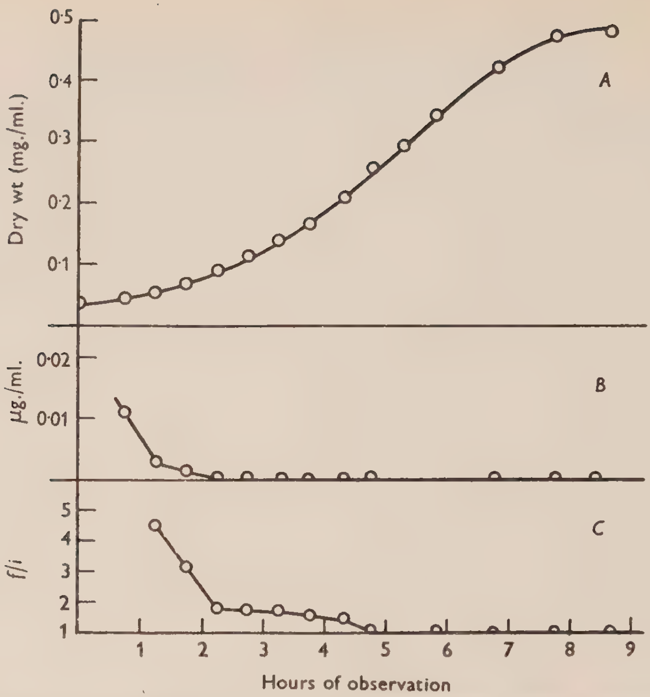

Fig. 2. Nicotinic acid content of medium during growth. The culture contained initially 0·03 µg. nicotinic acid/ml. *A*, growth curve; *B*, residual nicotinic acid in supernatant fluid of centrifuged samples; *C*, ability of cells collected at times shown to grow on transference to nicotinic acid-free medium, expressed as ratio of final dry wt./initial dry wt. (*f*/*i*).

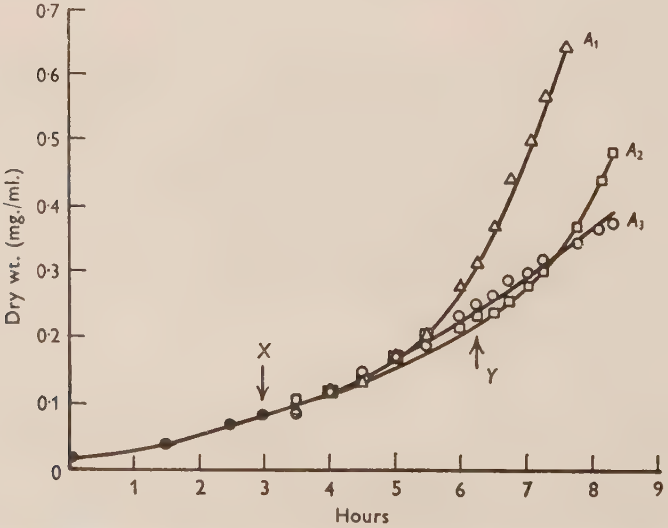

Fig. 3. Effect of adding extra nicotinic acid to a culture which contained initially 0·02 µg. nicotinic acid/ml. (●—●). At *X*, the culture was divided into three parts, A_1 (△—△), A_2, (□—□) and A_3, (○—○), 0·30 ml. 0·001 M nicotinic acid was added to A_1; at *Y*, 0·30 ml. 0·001 M nicotinic acid was added to A_2.

autoclaving; on subsequent assay, 95 % was recovered. The figures for nicotinic acid per unit cell mass show that during and after the linear phase of growth there was a progressive dilution of the nicotinic acid in the growing mass of protoplasm.

Table 1. *Recovery of nicotinic acid from* P. vulgaris *cells*

mg. cells/ 200 ml. culture	Nicotinic acid initially present in medium recovered from cells (%)	mμ. moles nicotinic acid/mg. dry wt. cells
38	57	0·73
41	53	0·63
50	57	0·56
50	53	0·52
102	54	0·26
109	54	0·24
124	55	0·22

These estimations were made on a series of similar cultures each containing initially 0·03 μg. nicotinic acid/ml. medium.

A few estimations were made of the nicotinic acid content of cells which had been grown in the presence of an excess of nicotinic acid (2·4 μg./ml.) and values up to 6 mμ moles/mg. dry wt. were found.

Oxygen uptake of growing cultures

The overall rate of oxygen uptake of a culture growing in a manometer vessel was constant during the period of linear growth (Fig. 4), the Q_{O_2} falling from 330 to 198 during the first 2 hr. of this phase. After the period shown in Fig. 4 the rate of oxygen uptake decreased very gradually. A further sample of culture was transferred from the main culture flask to a manometer vessel 24 hr. after the end of *B* and its oxygen uptake was measured for 5 hr. (curve *C*, Fig. 4). By the time this sample was taken the dry weight was stationary at 0·55 mg./ml., and the Q_{O_2} was found to be 56.

In a further experiment, the effect on oxygen uptake of adding extra nicotinic acid (0·20 ml. of $2·5 \times 10^{-5}$ M) to a 2·6 ml. sample of culture growing in the linear phase was observed. There was a prompt increase in rate of oxygen uptake, the Q_{O_2} rising from 208 to 300 in 10 min.

DISCUSSION

It is possible to interpret the curves of growth and of rates of oxygen uptake as showing that, after assimilation of all the nicotinic acid from the medium, the amount of active nicotinic acid derivatives formed within the cells is sufficient to maintain the rate of protoplasmic synthesis constant for a definite period. Our results have shown that at the time when nicotinic acid disappears from the medium exponential growth ceases and is followed by a linear growth phase during which the rate of oxygen uptake is constant. The constant rate of oxygen uptake under conditions where only nicotinic acid is limiting for growth is consistent with the hypothesis that there is a constant amount of some catalytic factor controlling the rate of oxygen uptake within the culture. Therefore, in so far as the organism has no other means of deriving energy from the environment, a linear growth phase would be expected. An analogous example of linear growth, in a streptomycin-requiring strain of *Bacillus cereus* deprived of streptomycin was reported by Schaeffer (1950), and Monod (1949) has discussed the significance of linear growth. It is, however, possible that in a culture whose growth is being limited by lack of nicotinic acid, the derivatives are being broken down, but are also being resynthesized from their breakdown products, so that for a time corresponding to the linear growth phase the rates of breakdown and resynthesis are balanced. McIlwain & Hughes (1948) showed that some bacteria actively decompose cozymase and that different organisms vary in their ability to resynthesize it. Nicotinamide and nicotinic acid may both occur as breakdown products of cozymase and from these Proteus can effect the resynthesis.

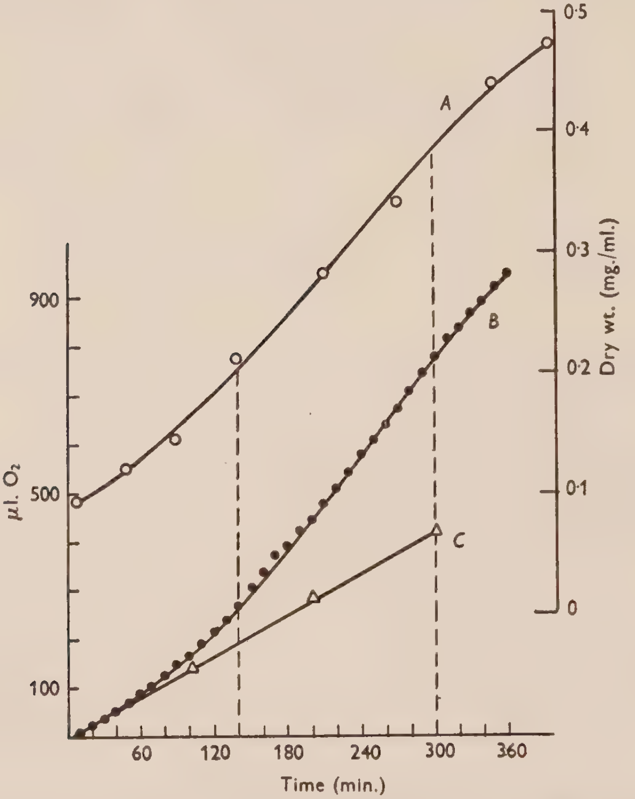

Fig. 4. Rate of oxygen uptake of a culture containing initially 0·03 μg. nicotinic acid/ml. *A*, growth curve (○—○); *B*, rate of O_2 uptake of a sample corresponding to *A* (●—●); *C*, rate of O_2 uptake of a sample of the same culture collected 24 hr. later (△—△).

Linear growth lasts in our cultures for 2·5–3 hr., and it is clear that its cessation might be due to the interplay of several factors, such as gradual irreversible inactivation of some of the coenzymes (see Morel, 1941), and dilution, to below a critical threshold concentration, of the nicotinic acid derivatives in the growing mass of protoplasm. It is possible that for protoplasmic synthesis to occur, certain structural relationships between coenzyme-requiring systems within the cell are necessary. Progressive separation, during growth, of active centres containing coenzyme might, therefore, lead to decrease in rate of protoplasmic synthesis even if the total amount of active coenzyme within the culture remained constant. Moreover, some apoenzymes may have a greater affinity than others for a particular coenzyme, so that with limitation of coenzyme supply the balance of distribution among the apoenzymes might be altered.

Failure to recover from the cells all the nicotinic acid originally added to the culture might possibly result from firm binding of some nicotinic acid to cell constituents so that it was not released by the methods used; in addition, part of the nicotinic acid might have been completely inactivated during growth. There is, however, some evidence that derivatives other than the pyridine nucleotides may play a part in the metabolism of Proteus (McIlwain, 1940; Saunders, Dorfman & Koser, 1941). The apparent loss of nicotinic acid in the present experiments might be at least partly explained on this basis if the unknown derivatives were not estimated by the methods used.

The continued exponential increase in numbers of bacteria during the linear growth phase of bacterial protoplasm shows that, in the present instance, the rate of cell division is less immediately affected by shortage of nicotinic acid than is the rate of protoplasmic synthesis. This dissociation between the two rates inevitably leads to progressive decrease in cell size, a phenomenon well known to occur during the later growth phases (Henrici, 1928).

We are indebted to Dr M. R. Pollock for suggesting the problem to us and for much advice during the course of the work. We also wish to thank Miss L. Hopkinson for her technical assistance and Miss K. de Bouk for advice on the assay of nicotinic acid.

REFERENCES

Barton-Wright, E. C. (1946). *Practical Methods for the Microbiological Assay of the Vitamin B Complex and Essential Amino-acids.* London: Ashe Laboratories, Ltd.

Fildes, P. (1938). The growth of *Proteus* on ammonium lactate plus nicotinic acid. *Brit. J. exp. Path.* **19**, 239.

Henrici, A. T. (1928). *Morphologic Variation and the Rate of Growth of Bacteria.* Springfield, Ill.: Charles C. Thomas.

McIlwain, H. (1940). Pyridine-3-sulphonic acid and its amide as inhibitors of bacterial growth. *Brit. J. exp. Path.* **21**, 136.

McIlwain, H. & Hughes, D. E. (1948). Decomposition and synthesis of cozymase by bacteria. *Biochem. J.* **43**, 60.

Miles, A. A. & Misra, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

Miller, L. & Houghton, J. A. (1945). The micro-kjeldahl determination of the nitrogen content of amino-acids and proteins. *J. biol. Chem.* **159**, 373.

Monod, J. (1949). The growth of bacterial cultures. *Ann. Rev. Microbiol.* **3**, 371.

Morel, M. (1941). L'usure de la nicotinamide chez *Proteus vulgaris*. *Ann. Inst. Pasteur*, **67**, 285.

Saunders, F., Dorfman, A. & Koser, S. A. (1941). The role of nicotinamide and related compounds in the metabolism of certain bacteria. *J. biol. Chem.* **138**, 69.

Schaeffer, P. (1950). Croissance et respiration d'une souche streptomycino-exigeante de *Bacillus cereus* privée de l'antibiotique-facteur de croissance. *Ann. Inst. Pasteur*, **78**, 624.

Sykes, J. A. & Reed, R. (1949). The control of the swarming of *Proteus vulgaris* by boric acid. *J. gen. Microbiol.* **3**, 117.

Wilson, G. S. (1922). The proportion of viable bacteria in young cultures with especial reference to the technique employed in counting. *J. Bact.* **7**, 405.

Wright, H. D. (1933). The importance of adequate reduction of peptone in the preparation of media for the pneumococcus and other organisms. *J. Path. Bact.* **37**, 257.

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The Influence of Temperature on the Motility of *Pasteurella pseudotuberculosis*

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SUMMARY: Grown at 22° *Pasteurella pseudotuberculosis* was motile and had flagella and H antigen; grown at 37° it was non-motile and had neither flagella nor H antigen. The change occurred over a narrow range of temperature about 30°. Cells grown at 37° when transferred to 22° developed flagella without cell division; cells grown at 22° when transferred to 37° became non-motile, due to an unexplained adverse effect indicated by a high death-rate. It appeared that different metabolic processes were used at the two temperatures; it was not necessary to postulate the selection of a naturally occurring mutant and no evidence was obtained for the existence of an extracellular enzyme which would destroy flagella at 37°.

In addition to the 'short-term' effect there was a 'long-term' effect of temperature shown by a difference in degree of motility and rate of growth (at 22°) of cultures grown for several months at 22 or 37°. This may have been an extension of the 'short-term' effect, a period of training being required for the optimal functioning of the metabolic processes concerned. The long-term effect could be reversed by prolonged cultivation of the 22° substrain at 37° and vice versa.

Preliminary experiments indicated that virulence was affected by the temperature of cultivation.

A survey of the literature shows fairly general agreement that *Pasteurella pseudotuberculosis* is non-motile at 37° but motile at about 22° (Arkwright, 1927; Boquet, 1937; Bhatnagar, 1940). However, Boquet noted that two strains became permanently non-motile, even at 20°, after culture at 37° for several years; one of Arkwright's strains was slightly motile at 37°; Weitzenberg (1935) observed a few short granular flagella on organisms grown at 37°. Schütze (1932) showed that all four types of *Past. pseudotuberculosis*, based on somatic antigens, produced a common flagellar antigen at room temperature. Boquet claimed that a flagellar antigen was produced at 37°, but gave no convincing proof.

The object of the present work was to explain the mechanism by which the temperature of incubation affected the production of flagella.

MATERIALS AND METHODS

A single strain (134) of *Past. pseudotuberculosis* which had been isolated from a guinea-pig and dried was used for the main experimental work. By subculture twice a week for several months in Craigie tubes at 22° and on agar slopes at 37°, two new substrains were produced, referred to as 134/22 and 134/37. They were maintained similarly, the medium in each case being prepared from tryptic digest broth. The parent strain and the substrains were alike in not fermenting lactose or sucrose; they produced acid (without gas) from glucose and mannitol in 24 hr., and from salicin in 5–7 days. Indole was not produced.

The strain was pathogenic for mice and guinea-pigs by intraperitoneal injection of broth cultures but its virulence was low; subcutaneous injection produced a non-progressive local lesion which became sterile after a few weeks. It was not pathogenic for white rats.

Other strains were obtained from the National Collection of Type Cultures.

To detect motility broth cultures were examined in a 'hanging-drop'.

The *staining technique* for flagella was developed from a modification of Gray's method (Conn & Wolfe, 1938) and yielded clean preparations with deeply stained flagella:—

Stand new slides in concentrated nitric acid for at least 24 hr.; wash in distilled water; rinse in ethanol; pass them through the Bunsen flame until it turns slightly orange; cool gradually on an asbestos mat, to minimize breakage.

With a cool loop, transfer a little growth from an overnight agar slope culture to a few ml. of sterile distilled water; allow to stand for 5–10 min.; put a loopful on a slide, and spread with the edge of another slide; allow to dry without heating. By using the deposit from a centrifuged overnight broth culture (as suggested by Leifson, 1930), allowing this to stand in water for only 2–3 min. before smearing, a better result may be obtained. The agar slope, or broth, should be inoculated with motile organisms from a Craigie tube.

Apply cold mordant for 10–15 min.; wash well in running water; blot gently, and dry in the air without heating; apply cold stain for 7–10 min; wash, and blot dry.

Mordant. Mix 5 ml. saturated aqueous potassium alum, 2 ml. saturated aqueous mercuric chloride, 2 ml. 20 % (w/v) aqueous tannic acid, and 0·4 ml. saturated ethanolic basic fuchsin; allow to stand for 2 hr.; filter twice through Whatman No. 1 filter-paper and use immediately.

Stain. Heat 15 g. phenol with 3 g. basic fuchsin on a water-bath; when completely dissolved, add 30 ml. ethanol; mix thoroughly; add 170 ml. distilled water; leave for at least 24 hr.; filter 3 times through Whatman No. 1 filter-paper just before use.

RESULTS

Motility and flagellation. Broth cultures grown for 48 hr. at 22° showed a general turbidity and fine deposit, and contained short actively motile rods. Similar cultures grown at 37° had a granular deposit, a surface pellicle, and a few scattered floccules, but no general turbidity; they consisted of longer non-motile rods. Stained preparations of agar slope cultures grown at 22° revealed organisms with one to six peritrichate flagella, more commonly only one or two (Pl. 1, fig. 1). It was later observed that stained deposits of broth cultures showed more numerous flagella (Pl. 1, fig. 2) and this method alone was subsequently used for their demonstration. Organisms grown at 37°, however, showed no flagella (Pl. 1, fig. 3). There is apparently a gradual change (over a small range of temperature about 30°) from active motility to complete absence of motility (Table 1).

Both substrains 134/37 and 134/22 grew round Craigie tubes at 22° in 1 to 4 days, depending upon the consistency of the medium. At 37° the

Table 1. *Effect of temperature on motility, morphology and growth in broth*

Temperature	Appearance of culture	
	Macroscopic	Microscopic
25°	Turbid	80% actively motile: mostly short bacilli, single or in pairs
27°	Turbid	80 % actively motile: mostly short bacilli, single or in pairs
29°	Diffuse floccules	50 % slowly motile: many long bacilli and chains
31·5°	Deposit and few floccules	One motile cell seen (very sluggish) mostly long bacilli and long chains
34°	Deposit and few floccules	Non-motile: mostly long bacilli and long chains

growth of strain 134/37 was confined to the line of inoculation, whilst strain 134/22 produced a more diffuse growth in the upper part of the medium within the central glass tube, but never reached the surface outside. This inability to grow round a Craigie tube at 37°, even after 2 weeks' incubation in a tube which had been sealed to prevent evaporation, provides further evidence in favour of a complete absence of motility. The diffuse growth of strain 134/22 was obtained even when the Craigie tube was warmed to 37° before inoculation, the inference being that bacteria in the inoculum retained their motility for a time at 37° and spread some way into the medium where they grew. Such temporary maintenance of motility at 37° was again apparent in later experiments.

H antigen. Rabbits were immunized with flagellated and non-flagellated organisms grown at 22 and 37° respectively. The growth from 48 hr. cultures on blood agar plates was suspended in normal saline containing 0·1 % (v/v) formalin. After standing at 4° until sterile, the suspensions were diluted to about 5×10^8 organisms/ml., and stored at 4°. Each rabbit received 0·5 ml. suspension intravenously on three successive days in the first week, and three doses of 1·0 ml. in each of the second and third weeks. Samples of blood were taken at weekly intervals before, during, and after the course of injections, and the sera, preserved with 0·5 % (w/v) phenol, were stored at 4°.

Suspensions for agglutination were made from cultures grown at 22°. The 'H' suspensions were made by diluting twofold the one used for immunization. The 'O' suspensions were made by boiling saline suspensions for 2 hr. and diluting them to the same density. The instability of suspensions of *Past. pseudotuberculosis* from cultures grown at 37° was recorded by Arkwright (1927). We were able to produce a relatively stable suspension from such cultures by shaking before boiling, but the tendency to auto-agglutination was not completely removed and the agglutination end-points were consequently less distinct than with organisms grown at 22°. The titres of H and O agglutinins were obtained by using Dreyer tubes, held overnight at 56° in a water-bath. The results are shown in Table 2. Sera made against organisms grown at 22° contained both H and O agglutinins, whilst those against organisms grown at 37° contained only O agglutinins. A similar absence of H agglutinin was found in the sera of rabbits immunized with a formalin killed broth culture grown at 37°.

Table 2. *Agglutination titres of rabbit sera against H and O antigens*

Serum: ...	...	A		B		C		D	
Antigen: ...	...	H	O	H	O	H	O	H	O
Rabbit no.	Immunized with organisms grown at								
1	22°	0	0	5,000	500	10,000	2,500	20,000	5,000
2	22°	0	0	2,500	0	20,000	125	50,000	5,000
3	22°	0	0	125	25	2,500	1,000	10,000	2,500
4	37°	0	0	0	25	0	125	0	2,500
5	37°	0	0	0	0	0	250	0	1,000
6	37°	0	0	0	25	0	250	0	1,000

A, serum taken before immunization; B, serum taken after first series of three injections; C, serum taken after second series of three injections; D, serum taken after third series of three injections.

Although these results are highly suggestive of an absence of H antigen in organisms grown at 37°, confirmation was sought by the absorption of immune serum. Sera with high titres of both H and O agglutinins were pooled, diluted 1/5, mixed with equal volumes of very heavy suspensions of organisms grown for 3 days at 22 or 37°, and left in contact overnight at 22 and 37° respectively. The titres of these absorbed sera against H and O suspensions were as follows:

	H	O
Serum absorbed with living 37° suspension	10,000	<25
Serum absorbed with formolized 37° suspension	10,000	1000
Control kept at 37°	10,000	2500
Serum absorbed with living 22° suspension	<25	<25
Serum absorbed with formolized 22° suspension	25 (tr.)	1000
Control kept at 22°	20,000	1000

Organisms grown at 22° absorbed both H and O agglutinins while those grown at 37° absorbed only O agglutinins. Treatment with formalin reduced the capacity of the organisms to absorb O agglutinin.

These experiments show that organisms grown at 22° were motile, possessed peritrichate flagella, gave rise to the production of H agglutinin in rabbits, and absorbed this antibody from immune serum. On the other hand, organisms grown at 37° were non-motile, possessed no flagella, did not cause the production of H agglutinin in rabbits, and were unable to absorb H agglutinin from immune serum. In each case there was complete correlation between the morphological and serological properties of this strain of *Past. pseudotuberculosis*.

There are several possible explanations for this difference associated with temperature:

(1) The establishment of different physico-chemical conditions in cultures at the two temperatures, e.g. different pH values.

(2) The production of an enzyme which at 37° would destroy the flagella or some substance essential for their synthesis (cf. streptococcal proteinase—Elliott, 1945).

(3) Mutation, and subsequent selection determined by the temperature of incubation.

(4) The use of different metabolic processes at the two temperatures. (Cf. amino-acid requirements of *Past. pestis*, Hills & Spurr, 1952.)

The influence of pH

Cultures in digest broth inoculated with motile organisms and incubated at 22 and at 37° were examined at intervals for motility, and the pH was estimated. The 22° cultures were actively motile; those grown at 37° showed only slight motility up to 24 hr. after which they were non-motile. At both temperatures, the pH showed an initial fall and subsequent rise (see Table 3). The changes in pH occurred faster at 37°, corresponding with more rapid growth, but otherwise they were similar at each temperature.

Table 3. *Changes in* pH *of cultures at* 22° *and at* 37°

Age of culture (hr.)	pH of culture grown at 22°	pH of culture grown at 37°
0	7·7	7·7
6	7·7	7·55
12	7·2	7·2
18	7·15	7·15
24	7·1	7·2
36	7·15	7·4
48	7·2	7·6
72	7·6	7·55

Each figure represents the average pH of two cultures.

In another experiment, digest broth was adjusted to pH 5·5, 6·0, 6·5, 7·0, 7·5, 8·0, 8·5, 9·0, 9·5. Substrains 134/22 and 134/37 were each used to inoculate a series of tubes covering the entire range which were incubated at 22 and 37° respectively. Overnight cultures at 22° were all motile; those at 37° were all non-motile. These experiments leave little doubt that the differences between organisms grown at the two temperatures are not due to differences in pH.

Search for a destructive enzyme

If the failure to produce flagella at 37° were due to the action of a destructive enzyme, such an enzyme might possibly be demonstrated in filtrates of 37° cultures. Broth cultures of strain 134 grown for 18 hr. at 22 and 37° were Seitz-filtered. Samples of each filtrate were re-inoculated and incubated for 18 hr. at both 22 and 37°. Of these four cultures, the two which were grown at 22° (one in a 22° filtrate, the other in a 37° filtrate) were actively motile and showed no apparent difference in flagellation. Those grown at 37° were non-motile. This experiment did not eliminate the possibility of an enzyme which acted effectively only at 37° or of one which was adsorbed on to a Seitz filter.

Evidence against these possibilities was provided by observations on tubes of broth which were inoculated with motile cells from a Craigie tube culture of strain 134/22. After 1 day at 37°, these cultures contained a few motile cells and stained deposits showed occasional flagellated organisms some of which were faintly stained. After 2 days at 37°, the cultures were non-motile, but stained deposits still showed some flagellated cells, though these were all faintly stained and presumably dead. Considering the relatively rapid fall in viability of 37° cultures, revealed in a later experiment, it seems likely that these flagellated cells were part of the inoculum and that they died at 37° without destruction of their flagella. In view of the enormous preponderance of non-flagellated cells in these cultures the survival of a few flagella renders most unlikely the possibility that the non-flagellates produce an enzyme which can destroy flagella.

Although these experiments yield no evidence of an enzyme capable of destroying formed flagella, there remains the possibility of an enzyme which attacks at 37° some substance necessary for the formation of flagella. Such a mechanism would appear, however, to be closely related to the utilization of different synthetic pathways at the two temperatures, and this will be discussed later.

'Short-term' and 'long-term' influence of temperature

The problem was to determine whether a culture is motile at 22° as a result of the production of motile mutants, or whether a different metabolic process occurs at 22° which enables the bacteria to develop flagella. Some light was thrown on this problem by a study of the time-factor involved in the development of motility at 22° and the loss of motility at 37°.

Broth cultures of strains 134/22 and 134/37 were incubated at 22 and 37°. They were examined for motility after 1, 2 and 3 days, and on each occasion the culture grown at 37° was placed at 22° and re-examined 2 hr. later. The results are shown in Table 4. Strain 134/37 grown at 22° had a considerable

Table 4. *Motility of strain* 134/22 *and* 134/37 *at* 22° *and at* 37°

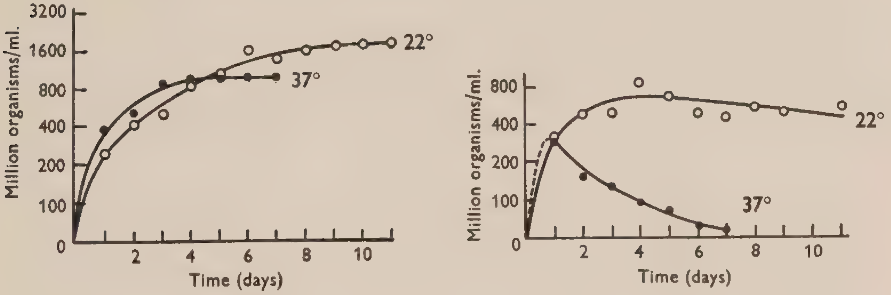
Strain	Temperature of incubation	1 day	2 days	3 days
134/22	22°	90 % actively motile	50 % actively motile	50 % actively motile
134/22	37°	Occasional cell very sluggishly motile—rotating with little progress	Non-motile	Non-motile
	+2 hr. at 22°	More cells motile, and more active	Occasional cell rotating with little progress	Non-motile
134/37	22°	50 % actively motile (not as fast as strain 134/22)	50 % actively motile	50 % actively motile
134/37	37°	Non-motile	Non-motile	Non-motile
	+2 hr. at 22°	Non-motile	Non-motile	Non-motile

degree of motility after 24 hr.; strain 134/22 grown at 37° for 24 hr. had only a few sluggishly motile cells, probably from the inoculum.

In addition to this immediate or 'short-term' effect of temperature there was a cumulative or 'long-term' effect. Strain 134/22 grown at 37° developed some motility 2 hr. after transferring it to 22°, whereas strain 134/37 required longer. After 24 hr. at 22° strain 134/22 was more actively motile than strain 134/37.

The mechanism of the short-term effect

If the effect of temperature was to alter enzymic activity, it might be possible to demonstrate this in resting cells. Therefore broth cultures grown at either 37 or 22° until they reached the stationary phase were kept at the opposite temperature. Under these conditions changes in motility could be observed when there was little or no multiplication of the organisms. Viable organisms were estimated by counting colonies on blood agar by the method of Miles & Misra (1938); total counts were made in a Thoma blood-counting chamber. Microscopic clumps which formed in broth cultures at 22°, and to a greater extent at 37°, were broken up, without lowering their viability, by rapid shaking in a vibrator. Trials indicated that the error in the viable count was unlikely to exceed ±15 %, whilst that of the total count was probably less than ±10 %; these figures refer to the relative counts of different cultures, not to the absolute values of the total and viable counts.



a *b*

Fig. 1. *a*, growth of *Past. pseudotuberculosis*, strain 134, in digest broth (total counts); *b*, growth of *Past. pseudotuberculosis*, strain 134, in digest broth (viable counts).

Digest broth cultures of strain 134 reached the stationary phase at 37° after 3–4 days; at 22° multiplication was active up to about 5 days but later was much slower (Fig. 1). An experiment based on these findings was made to test the effect of change of temperature on cells which were not dividing. Tubes of digest broth were inoculated with a motile culture from a Craigie tube. After incubation at 37° for 4 days (A) some tubes were transferred to 22° for 1 day (B); a control was incubated at 37° for 5 days (C). Other tubes were incubated at 22° for 5 days (D) and some of them transferred to 37° for 2 days (E); a control was kept at 22° for 7 days (F). Motility, flagella, total and viable counts are shown in Table 5.

Table 5. *Effect of change of temperature on cultures which have reached the stationary phase*

Culture	Time and temperature of incubation	Total count (million/ ml.)	Viable count (million/ ml.)	Hanging drop	Stained deposit
A	4 days at 37°	840	300	Non-motile	None flagellate
B	A+1 day at 22°	800	320	About 30–40 % motile	About 30 % flagellate (peritrichate)—all these deeply stained
C	A+1 day at 37°	840	280	Non-motile	None flagellate
D	5 days at 22°	990	625	About 40–50 % motile	About 70 % flagellate (peritrichate)—about one third of these pale
E	D+2 days at 37°	880	100	Less than 5 % motile (sluggish)	About 70 % flagellate —most of these pale (deeply stained cells had not more than three flagella: most had none or one)
F	D+2 days at 22°	960	500	About 40–50 % motile	About 70 % flagellate (peritrichate)—about half of these pale

About one-third of the bacteria in B became motile during 24 hr. at 22° without a significant change in the total or viable count. That this was a result of temperature is shown by C; cells which were non-motile at 37° became motile at 22°, a finding that would explain the temperature effect without recourse to mutation. The only other possibility was that the motile cells were newly formed as replacements for lysed cells, but there was no evidence to support this view: examination of hanging drops and stained films did not show any abnormal cells indicative of autolytic processes, and, moreover, many of the motile cells were long rods, the form usually found in cultures grown at 37°.

Conversely, cells which were motile when grown at 22° (D) became non-motile after a further 2 days at 37° (E), a change that did not occur at 22° (F) and was therefore a temperature effect.

This loss of motility when flagellated cells grown at 22° were kept for 2 days at 37° was associated with fall in viable count, inability of large numbers of cells to stain with normal intensity (Table 5) and, in addition, the presence of large numbers of poorly refractile organisms, 'ghost-cells' seen in hanging-drop preparations, indications of an adverse effect of a temperature of 37° on the viability of the cell in the medium employed. Loss of motility was not due to loss of flagella but probably was a manifestation, perhaps an early one, of conditions found in digest broth at 37°, not operative at 22°, which were unfavourable to survival of the bacteria. The nature of these conditions and the mechanism of their effect are not known. It is probable, however, that the factors which suppress development of flagella at 37° also bring about the loss of motility when motile cells grown at 22° are kept at 37° and account for the high death-rate of bacteria at 37°. The temperature effect which suppresses the production of flagella or abolishes motility in flagellated organisms is not due to mutation; a more likely explanation is that certain metabolic processes are conditioned by temperature. Thus the short-term effects of temperature, both 22 and 37°, have a similar explanation; the metabolic processes at these two temperatures appear to be different.

It may be noted that motility in culture E grown at 22° and kept at 37° (Table 5) was not completely abolished and that some of the cells were deeply stained and had flagella. These cells were probably some which had developed at 22°; there was no evidence that new cells were produced in this culture at 37°. However the possibility cannot be completely excluded that a few flagellated cells may have been formed at 37°. A motile strain inoculated into a Craigie tube and incubated at 37° grew through the medium for a time; perhaps some motile cells retained for a time some stored-up material which enabled them to use their flagella at 37° and even develop a few new flagella but that this soon came to an end. Two-day cultures in broth at 37° were non-motile and 48 hr. cultures on blood agar at 37° had no detectable H antigen.

The development of flagella at 22° without cell division was also observed in nutrient broth, measuring density with a nephelometer. Cultures were inoculated with motile organisms, incubated for 2 days at 37°, and overnight at 22°. In one case about 15 % of the cells had become motile without increase in opacity; in the other there was an increased opacity of about 4 % but about 25 % had become motile. Similar results were obtained with four other strains of *Past. pseudotuberculosis* belonging to different serological types. Three further strains required four passages through Craigie tubes before becoming actively motile at 22°, and then gave similar results.

The long-term effect

A difference in the properties of the two substrains, induced by repeated subculture at 22 and 37° was demonstrated by comparing the rates of progress of their growth in Craigie tubes at 22°. Overnight broth cultures of the two substrains 134/22 and 134/37 of approximately equal density, were used. Each was grown at 22 and 37° and inoculated with a straight wire into five Craigie tubes which were incubated at 22°. All these tubes were similar in size. The progress of the growth down the central tube and up the outer part of the medium was recorded daily, the measurements of the five tubes of each series being averaged, as shown in Table 6 and Fig. 2. It is clear that the two substrains, as a result of prolonged cultivation at different temperatures, differed in their ability to grow and produce motile organisms at 22° and that this difference depended more on properties acquired by long-term cultivation than the conditions of the culture immediately preceding their inoculation. Substrain 134/22, accustomed to grow at a temperature suitable for motility, made faster progress through a Craigie tube at 22° than substrain 134/37, accustomed to grow at a temperature which precluded the formation of flagella. This was so whether the culture used as inoculum was motile or non-motile.

The long-term effect was also demonstrated by a difference in the rate of growth in digest broth, using a nephelometer. Substrains 134/22 and 134/37 were equal at 37°, but at 22° substrain 134/22 grew considerably faster (Fig. 3*a*, *b*).

Table 6. *Rate of progress in Craigie tubes at 22°*

		Distance in mm. from point of inoculation				
Group	Tube	1 day	2 days	3 days	4 days	5 days
I	1	13	37	72+	—	—
	2	15	38	62	70+	—
	3	10	28	Broken	—	—
	4	17	34	64+	—	—
	5	13	33	58	61+	—
	Av.	14	34	64+	—	—
II	1	10	29	57	68+	—
	2	12	34	55	64+	—
	3	13	34	62	64+	—
	4	12	35	60	66+	—
	5	9	24	54	65+	—
	Av.	11	31	58	—	—
III	1	9	21	37	54	69+
	2	13	25	39	59	73+
	3	11	22	36	66+	—
	4	8	21	31	60+	—
	5	8	21	34	65+	—
	Av.	10	22	35	61+	—
IV	1	5	16	36	51	68+
	2	6	35	47	67+	—
	3	7	14	34	45	64+
	4	8	20	35	62+	—
	5	6	20	33	62+	—
	Av.	6	21	37	57+	—

I, inoculated with strain 134/22 grown overnight at 22° (motile inoculum); II, inoculated with strain 134/22 grown overnight at 37° (inoculum almost non-motile); III, inoculated with strain 134/37 grown overnight at 22° (motile inoculum); IV, inoculated with strain 134/37 grown overnight at 37° (non-motile inoculum); + indicates that the surface of the outer part of the medium had been reached.

Preliminary experiments have shown that the long-term effect is reversible. If substrain 134/37 is grown continually at 22° it assumes the properties described above for substrain 134/22; similarly, substrain 134/22 may be made to acquire the properties of substrain 134/37 by cultivating it for a period at 37°.

The probable explanation of this long-term effect is that it is a continuation of the short-term effect of temperature, the organism being trained, or becoming adapted, to use different metabolic processes more efficiently after repeated subculture. As the short-term effect can be explained apart from the gradual selection of a naturally occurring mutant, it would seem unnecessary to introduce such a postulate to explain the long-term effect.

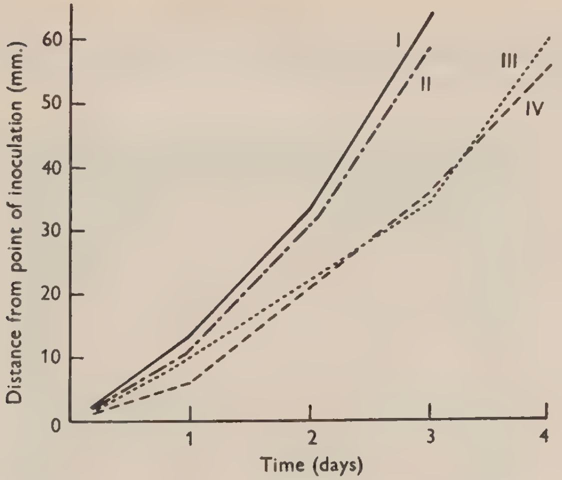
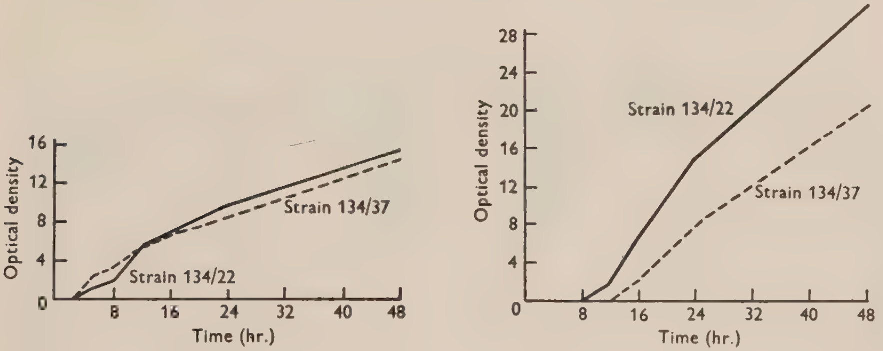


Fig. 2. Progress of *Past. pseudotuberculosis* in Craigie tubes at 22°. I, inoculated from overnight broth culture of strain 134/22 grown at 22° (motile inoculum); II, inoculated from overnight broth culture of strain 134/22 grown at 37° (inoculum almost non-motile); III, inoculated from overnight broth culture of strain 134/37 grown at 22° (motile inoculum); IV, inoculated from overnight broth culture of strain 134/37 grown at 37° (non-motile inoculum).



a *b*

Fig. 3. *a*, growth of *Past. pseudotuberculosis* in digest broth at 37°; *b*, growth of *Past. pseudotuberculosis* in digest broth at 22°.

Virulence for mice

Preliminary experiments have indicated that organisms grown in digest broth at 22° were more virulent for mice than those grown at 37°. This confirms one of the findings of Boquet (1937). It would seem unlikely from analogy that this phenomenon is related to the presence or absence of flagella. Further investigations are in progress.

We are grateful to Mr F. Ward and Mr H. W. Gaze for the photographs.

REFERENCES

ARKWRIGHT, J. A. (1927). The importance of motility of bacteria in classification and diagnosis, with special reference to *B. pseudotuberculosis rodentium*. *Lancet*, i, 13.

BHATNAGAR, S. S. (1940). Bacteriological studies on *Past. pestis* and *Past. pseudotuberculosis*. *Indian J. med. Res.* **28**, 1.

BOQUET, P. (1937). Recherches expérimentales sur la pseudo-tuberculose des rongeurs. *Ann. Inst. Pasteur*, **59**, 341.

CONN, H. J. & WOLFE, G. E. (1938). Flagella staining as a routine test for bacteria. *J. Bact.* **36**, 517.

ELLIOTT, S. D. (1945). A proteolytic enzyme produced by group A streptococci with special reference to its effect on the type-specific M antigen. *J. exp. Med.* **81**, 573.

HILLS, the late G. M. & SPURR, E. D. (1952). The effect of temperature on the nutritional requirements of *Pasteurella pestis*. *J. gen. Microbiol.* **6**, 64.

LEIFSON, E. (1930). A method of staining bacterial flagella and capsules together with a study of the origin of flagella. *J. Bact.* **20**, 203.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

SCHÜTZE, H. (1932). Studies in *B. pestis* antigens. *Brit. J. exp. Path.* **13**, 284.

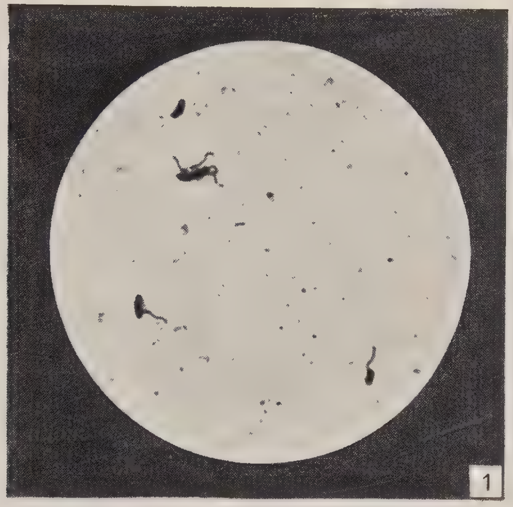
WEITZENBERG, R. (1935). Über die Beweglichkeit des *Bac. pseudotuberculosis rodentium* (A. Pfeiffer). *Zbl. Bakt.* **133**, 343.

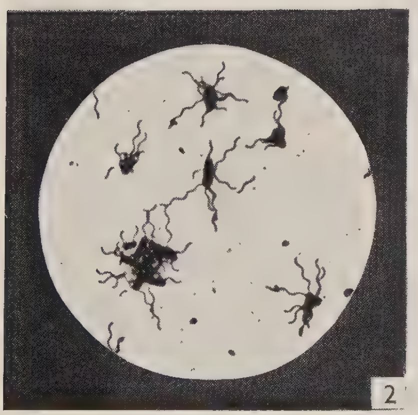
EXPLANATION OF PLATE

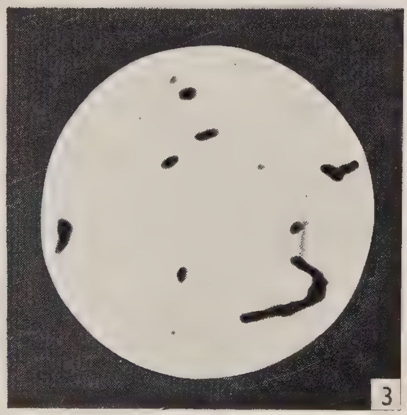
Photomicrographs of *Past. pseudotuberculosis* stained to show flagella.

Fig. 1. Grown at 22° on nutrient agar. ×600.
Fig. 2. Grown at 22° in broth. ×700.
Fig. 3. Grown at 37° in broth. ×700.

(*Received* 9 *February* 1952)







N. W. Preston & H. B. Maitland—Motility of *Pasteurella pseudotuberculosis*. Plate 1

HUMPHREY, J. H. & LIGHTBOWN, J. W. (1952). *J. gen. Microbiol.* 7, 129–143

A General Theory for Plate Assay of Antibiotics with some Practical Applications

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SUMMARY: The distribution of antibiotic (or other substance) in the agar around a container or around a hole in a punch-plate can be expressed theoretically by an equation involving: the initial quantity of antibiotic, the depth of the agar layer, the diffusion constant, the concentration at a given distance from the container, and the time of diffusion. The validity of the equation was confirmed by measurement of the diffusion constants of penicillin, streptomycin and aureomycin, and of the critical concentration of these substances required to inhibit test organisms, followed by the use of the values so obtained to predict the sizes of the inhibition zones produced experimentally by these antibiotics after varying periods of diffusion.

The theory predicts that the square of the inhibition zone diameter will be proportional to the logarithm of the antibiotic concentration. This relationship was found to hold, when accurate assays were made, for a number of antibiotics but not for penicillin when tested with *Bacillus subtilis*. The most important factor determining the slope of the dose-response curve under given conditions is the diffusion constant of the antibiotic. The slope can, however, be increased by prolonging the time allowed for diffusion.

Particular factors which affected both the sharpness of the zone edge and the nature of the dose-response curve were production of small amounts of penicillinase by strains of *B. subtilis* used for penicillin assay, and uptake of streptomycin by organisms used for streptomycin assay. Measurements of adsorption of streptomycin by *B. subtilis*, *B. pumilus* and by *Staphylococcus aureus* were made, and were shown to fit equations of the Freundlich isotherm type.

Although very good plate assay methods are available for penicillin and streptomycin, those described for the newer antibiotics are less satisfactory owing to a tendency for the zone edges to be poorly defined and to shift their position as incubation of the plates is continued. Unless a valid theoretical treatment of the nature of diffusion of an antibiotic from the cup into the surrounding agar gel is available, attempts to investigate the factors governing the nature of the zone edge and the slope of the dose-response curve for new antibiotics will be empirical and time-consuming. The only theoretical treatment of this problem of which we were aware was that given by Cooper & Woodman (1946), who discussed diffusion of antiseptics and of penicillin from a Heatley cup into agar, and claimed good agreement between predicted zone diameters and those which they observed in practice. They used, however, a formula derived from considerations of linear, not radial, diffusion, and assumed that the concentration of antibiotic in the cup was constant during the first 8 hr. These assumptions do not hold in plate assays as often performed, and we decided therefore to investigate the theoretical treatment from first principles. With the aid of certain assumptions, which are discussed below, a theoretical expression was derived, differing somewhat from that given by

Cooper & Woodman; its validity was tested in various ways. These tests revealed that in penicillin and streptomycin assays, under certain conditions, there is a sudden fall in concentration of antibiotic at the zone boundary, which results in a very sharp edge. Tests were also made to discover the reason for this boundary effect.

METHODS

Fish spine beads, size no. 2 (obtainable from Messrs Taylor, Tunnicliffe and Co., 125 High Holborn, London, W.C. 1) were used (Stewart & Thorpe (to be published)). These beads are very uniform, and may be assumed to hold on the average 0·025 ml.

Assay trays. Large flat trays, 10 × 10 in. accommodating 64 beads were used (Kantorowicz, 1951).

Organisms. Spores of *B. subtilis*, I.C.I. strain (NCTC 8236) and *B. pumilus* Mill Hill strain (NCTC 8241) were used for most assays. Suspensions were incorporated into molten agar at 60–70°, at a density of approximately 3×10^7 and 6×10^7 viable spores/ml. of medium respectively.

For certain experiments suspensions of *Staphylococcus aureus* (NCTC 7447) or of *Sarcina lutea* (NCTC 8340) were used. Suspensions were made with a density equivalent to Brown's tube no. 6 (Burroughs Wellcome Ltd.) and to ten times this strength respectively, and were incorporated into molten agar at 45° at a concentration of 1% (v/v).

The strains used had been subjected to several single colony isolations and were stored freeze-dried, except for the *B. subtilis* and *B. pumilus* strains which were stored at 2° as spore suspensions.

Media. Experiments with penicillin and aureomycin were performed with a Lemco Marmite peptone agar pH 7 when *B. subtilis* I.C.I. strain was used, and with the enriched seed layer medium prescribed by the Food and Drugs Administration of the U.S. Federal Security Agency (1951) when other organisms were used.

Experiments with streptomycin and dihydrostreptomycin were carried out in nutrient agar containing peptone, beef extract and yeast extract pH 7·8.

Difco agar 2 % was used in all media.

Zone diameters were measured on a screen after × 10 magnification. Under the conditions of our assays the coefficient of variation of the diameter of a single zone was repeatedly found to be approximately 1 %.

Diffusion constants. These were measured by the method of Friedman & Kraemer (1930). In this method a layer of agar gel is formed in a cylindrical container, and an equal volume of liquid is placed above it. The liquid layer is stirred continuously. Initially the substance whose diffusion constant is to be measured is either wholly in the agar layer or wholly in the liquid layer. By measurement of the change in concentration in the liquid layer, and the use of a theoretical expression derived by March & Weaver (1928) for this system, the diffusion constant can be obtained. It should be noted that the theoretical expression quoted by Friedman & Kraemer is wrong (although the curve which they give is correct), and the proper equation relating v, the fraction diffused across the boundary, and the diffusion constant k is as follows (taken from March & Weaver):

$$v=\tfrac{1}{2}-\Sigma(\pi^2Bi/4Zi^2)\ e^{-Zi^2T}, \tag{1}$$

where $T=kt/a^2$. In this expression a = depth of agar layer, k = diffusion constant, t = time, Z is given by the roots of $\tan Z+Z=0$. B is a function whose values are $B_0=0{\cdot}545$, $B_1=0{\cdot}748$, $B_2=0{\cdot}789$, $B_3=0{\cdot}799$, B_4 and higher terms $=0{\cdot}810$ $(=8/\pi^2)$.

This equation, expanded to 10 terms, becomes

$$v=\tfrac{1}{2}-[0{\cdot}327\,e^{-4{\cdot}117T}+0{\cdot}0766\,e^{-24{\cdot}14T}+0{\cdot}0306\,e^{-63{\cdot}68T}+0{\cdot}016\,e^{-123T}+0{\cdot}01\,e^{-200T}$$
$$+0{\cdot}0067\,e^{-300T}+0{\cdot}0048\,e^{-418T}+0{\cdot}0036\,e^{-556T}+0{\cdot}00279\,e^{-716T}+0{\cdot}00115\,e^{-896T}+\dots].$$

By the use of this expression a curve relating v and T can be drawn, from which the diffusion constant can be derived for values of $v>0{\cdot}05$. (If the expression is to be applied for lower values of v account must be taken of higher terms of the expansion.)

In our experiments the diffusion was measured under conditions as close as possible to those obtaining in the assay plates. The agar layer was made from nutrient agar which had been allowed to attain equilibrium with the nutrient broth used for the overlying liquid layer, by fragmentation of the agar, followed by 3 days contact at 2°. In the case of penicillin and dihydrostreptomycin the concentration in the small samples taken from the liquid layer was measured by an accurate biological method. In the case of aureomycin the concentration was measured colorimetrically, for which purpose concentrations of the order of 100–500 μg./ml. were required. These concentrations are greater than can be obtained in nutrient broth and therefore 1 % KH_2PO_4 was used in both layers. Control experiments were run to allow for possible inactivation of the antibiotics during the course of the experiment.

In order to ensure adhesion of the agar to the cylinder wall, the latter was coated with a layer of 'Perspex'.

EXPERIMENTAL

Theory of diffusion

All experiments were performed with cups consisting of the 'fish spine' insulator beads which are filled by capillarity, and whose effect is to place a uniform large drop on a uniform small area of the agar surface. Such a bead is shown in Fig. 1, which also shows the manner in which the contained liquid enters the agar layer. By the use of isotonic solutions of dyes we found that under average conditions a bead was empty in 2–3 hr., after which further spread occurred by simple diffusion. An approximate theoretical treatment can be derived on the assumption that a known quantity of antibiotic is placed in the agar at zero time in the form of an infinitely thin pencil, and that radial diffusion takes place from the pencil. Although this assumption is clearly not correct during the first 2 hr., it becomes increasingly true at later stages, and permits at least an approximate prediction of the behaviour of the diffusing substance.

We are indebted to Mr A. G. Liddiard of the Department of Theoretical Physics, King's College, London, for deriving the following expression which describes the theoretical behaviour of such a system:

$$\sigma = \frac{M}{4\pi hDt} e^{-r^2/4Dt}, \tag{2}$$

where σ = concentration of substance at a distance r from the centre, M = total quantity of dissolved substance, h = depth of agar layer, D = diffusion constant, t = time from start.

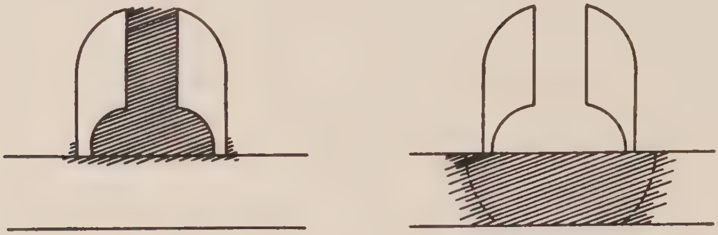

Fig. 1. Diagram illustrating movement of solution from a fish spine bead into the agar layer.

We found, after this work was completed, that Vesterdal (1947) had used a similar expression to predict the diffusion of penicillin out of the holes in a 'punch' plate, although he did not prove its validity in practice. When Heatley cups or dry paper disks are used, however, the expression might require modification.

The expression can be applied to the prediction of the behaviour of inhibition zones in antibiotic assays on the reasonable assumption that the zone edge appears where the concentration of antibiotic has a particular critical value at a definite time after beginning incubation. This period of time is the 'critical time', and will in general depend upon the growth characteristics of the test organism under the conditions of the assay and in certain circumstances upon the time interval between the appearance of the zone edge and its measurement.

Test of validity of the theoretical expression

The validity of the theory and the assumption made can be tested by attempting to deduce from it the zone diameters to be expected for different antibiotics after allowing various periods of diffusion before incubation. The theoretical expression (2) can be rewritten in the form:

$$r^2 = 9{\cdot}21Dt(\log M - \log 4\pi hDt\sigma), \tag{3}$$

from which r, the radius of the zone, may be evaluated if the other terms are known. The values of M and h can be fixed at will.

The 'critical' concentrations σ was determined experimentally by measuring the minimum inhibitory concentration of antibiotic in pour plates. A layer of agar medium seeded with the test organism was poured in a series of Petri dishes. When the agar was set, half was removed from each plate, and the corresponding spaces were filled by volumes of seeded agar identical in all respects with that removed, except that they contained graded concentrations of the antibiotic under test. The plates were incubated under conditions resembling, as far as possible, those used in our assays, and the growth in the 2 halves was compared. In the case of penicillin and streptomycin a fairly sharp limiting concentration could be distinguished, above which growth was almost entirely prevented. With aureomycin, however, although after 8 hr. incubation a sharp end-point could be distinguished, after 24 hr. incubation growth had occurred, albeit of diminishing intensity, in a number of higher concentrations. This was interpreted as being due to instability of aureomycin in nutrient agar at 35°, with consequent loss of its bacteriostatic effect. The results of our determinations are given in Table 1.

Table 1. *Minimum inhibiting concentrations of antibiotics in agar plates seeded with test organisms*

	Temp.		Minimum inhibiting concentrations	Time (hr.)
Penicillin	38°	*B. subtilis* I.C.I.	0·018 u./ml.	8–24
	28°	*Sarcina lutea*	0·008 u./ml.	16
Streptomycin	38°	*B. subtilis* I.C.I.	0·03 u./ml.	8–24
Dihydrostreptomycin	38°	*B. subtilis*	0·06 u./ml.	8–24
Aureomycin HCl	35°	*Staph. aureus*	0·04 μg./ml.	8
			c. 0·10 μg./ml.	24

The diffusion constants (*D*) were measured as described under Methods, and some results are recorded in Table 2. Most measurements were made at 4° because in subsequent experiments diffusion in the cold proved to be necessary to prevent growth of the test organism and to minimize inactivation of such antibiotics as are unstable under assay conditions. Diffusion constants are much affected by changes in the viscosity of water, and hence by changes of temperature, but corrections for this can be applied on the basis of tables available in *International Critical Tables* (1929).

Table 2. *Diffusion constants in* 2 % *agar*

	Temp.	Diffusion constant (cm.^{2}/hr.)
Penicillin	20°	0·011
Penicillin	37°*	0·016
Penicillin	4°*	0·0066
Aureomycin	4°	0·0075
Dihydrostreptomycin	4°	0·0053

* Calculated by comparison with tables of diffusion constants given in *International Critical Tables* (1929).

The critical time (i.e. the time after incubation is begun at which the zone size and edge are determined) was not easy to determine in practice. The problem was approached in two ways. In the first, which was used by Cooper & Woodman (1946), assay plates were poured containing seeded agar, and beads containing a constant amount of antibiotic solution were placed on them at varying intervals of time after incubation was begun. It was argued that beads placed after the critical time should show no inhibition zones. The drawback to this method lay in deciding whether to take account of small zones of inhibition which appeared even after general growth had occurred, and were associated with such high antibiotic concentrations that they were probably due to lysis. The second method consisted in placing many beads containing a constant concentration of antibiotic upon an assay plate. The plate was incubated at 37°, and from it at varying intervals of time, beads were removed, together with the agar immediately below and around them, by means of a cork borer. After the critical time removal of the bead may be expected not to affect the zone size. Estimates of the critical time obtained by the second method have always been about 1 hr. shorter than those by the first method, and for practical purposes we have taken a mean value. Since the concept of a sharp critical time is somewhat abstract, it is doubtful whether greater precision could be obtained.

With the values obtained it was possible to predict theoretically the zone diameters when antibiotics were allowed to diffuse for varying periods of time into seeded agar plates in the cold (i.e. without growth of the test organism) before incubation was begun. In order to permit sufficient replicate beads for accurate measurement at each time interval, it was necessary to use two assay plates in a single experiment. These were prepared as alike as possible, and one of the sets of replicate beads was repeated on each plate, as a check to ensure satisfactory duplication. In Fig. 2 are shown the experimental zone diameters observed at two levels of aureomycin concentration in relation to those predicted theoretically using the value for the diffusion constant obtained at 4°. Although failure to correct for the increased diffusion constant during the incubation period entails some error in the theoretical curves, it will not be great since the incubation period was short compared with the total period allowed for diffusion. It will be seen that the experimental points lie fairly close to the predicted curves, although a better fit is obtained with a slightly lower value for the critical concentration than that recorded in Table 1.

Fig. 3 shows a similar experiment, using penicillin with *B. subtilis* as test organism, conducted throughout at 37°. Because growth occurred throughout the experiment at this temperature the observation period was limited to 3¼ hr. By using short time intervals and many replicate beads we obtained enough experimental points to show that under these conditions also agreement with the theory was remarkably good, except when the period of diffusion was brief. Since in this part of the curve the time interval between placing beads on the agar and the appearance of inhibition zones was too short to allow complete entry of the fluid into the agar, the conditions assumed in the theory did not apply, and the aberrant points can be discounted.

Consequences of the theory

Regarding the theory as substantially valid we can deduce some practical consequences which will apply not only to inhibition zones produced by antibiotics but also to exhibition zones produced by growth factors.

(i) Fig. 4 shows some distribution curves of antibiotic in the agar around a bead, calculated for varying intervals of time. The figure in question relates to penicillin, but the general form of the curves applies to other substances, and it emphasizes how greatly the concentration at the centre exceeds that at the boundary. In the case of the diffusion assay of growth factors it is clear that there is little likelihood of organisms which grow inside the zone exhausting the growth factor to any significant extent.

(ii) From equation (3) it is apparent that under any given set of assay conditions r^2 should be proportional to log M.

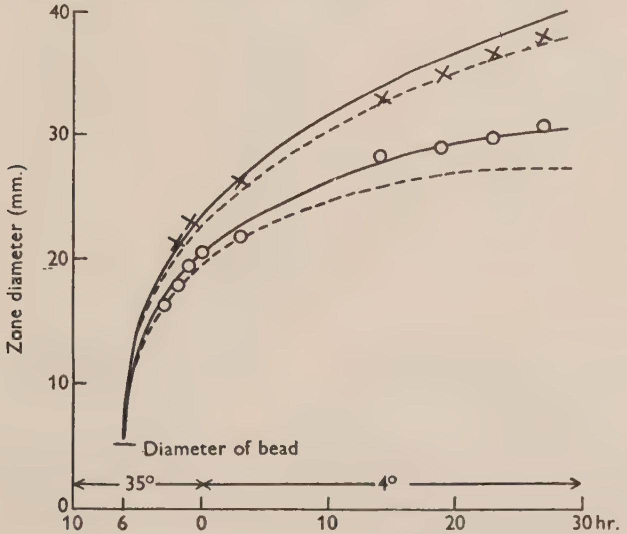

Fig. 2. Zone diameters observed after varying periods of diffusion in the cold followed by incubation at 35°, compared with diameters predicted theoretically. Antibiotic: aureomycin; test organism: *Staph. aureus.* × × and ○○ are experimental points for beads containing solutions of 25 and 6·25 μg./ml. respectively. Each point is the mean of 5 diameters, which were read after 8 hr. incubation. The theoretical curves are drawn from the expression

$$r^2 = 9{\cdot}21\,Dt(\log M - \log 4\pi hDt\sigma),$$

where $D = 0{\cdot}0075$ cm.²/hr., $h = 0{\cdot}4$ cm., and $M = 0{\cdot}625$ and $0{\cdot}156\,\mu$g. ——, curves when $\sigma = 0{\cdot}025\,\mu$g./ml.; ----, curves when $\sigma = 0{\cdot}035\,\mu$g./ml.

There has been much discussion as to whether the relationship between the log. of the antibiotic concentration and zone diameter is linear or whether a square term is involved. The general, although not the universally agreed, conclusion is that in penicillin assays the relationship is linear. It must be borne in mind, however, that a significant departure from a linear relationship will be noticed only if an assay is very accurate (in the sense that the coefficient of variation of a single zone is small and many replicates are used) or if a wide range of concentrations of antibiotic is used. We have found that when very accurate assays were performed (using 64 replicates at each dose level) the logarithm of the antibiotic concentration was more nearly proportional to the square of the zone diameter than to the zone diameter in the following assays: streptomycin and dihydrostreptomycin with *B. subtilis* as test organism;

aureomycin and terramycin with *B. pumilus*; penicillin with *Staph. aureus*. When penicillin was assayed with *B. subtilis* I.C.I. strain the zone diameter, and not its square, gave the best fit. A possible reason for this discrepancy, which may have wider application, is given below.

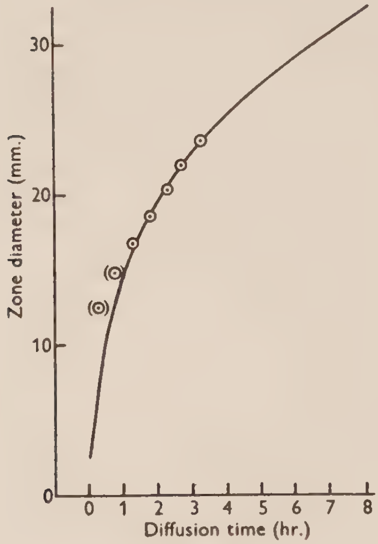

Fig. 3

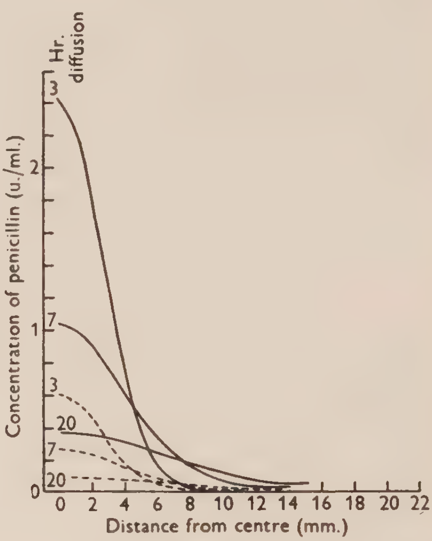

Fig. 4

Fig. 3. Zone diameters observed after varying periods of diffusion at 37°, compared with those predicted theoretically. Antibiotic: penicillin; test organism: *B. subtilis*. Each experimental point is the mean of 16 beads, containing a solution 16 u./ml. The theoretical curve was plotted from

$$r^2 = 9{\cdot}21Dt(\log M - \log 4\pi hDt\sigma),$$

where $D = 0{\cdot}016$ cm.²/hr., $M = 0{\cdot}4$ unit, $h = 0{\cdot}4$ cm., $\sigma = 0{\cdot}02$ u./ml.

Fig. 4. Curves illustrating the distribution of penicillin concentration in an agar layer depth 0·4 cm. around fish spine beads containing penicillin solution, after 3, 7 and 20 hr. diffusion at room temperature. ——, bead with 16 u./ml.; – – – –, bead with 4 u./ml. The critical concentration is 0·02 u./ml.

(iii) The sharpness of the edge of a zone, apart from effects due to lysis, will depend upon the range of antibiotic concentrations over which partial but not complete inhibition of growth occurs. Suppose that σ_2 is the critical concentration which permits no growth, and σ_1 the concentration which permits full growth of the test organism. Then over the range σ_1 to σ_2 a shading of growth will occur. Let r_1 and r_2 be the corresponding distances from the cup, so that the width of the edge is $r_1 - r_2$. From the expression (3) it can be shown that

$$r_1 - r_2 = \frac{9{\cdot}21Dt}{2R} \log \left(\frac{\sigma_2}{\sigma_1}\right),$$

where R is the mean of r_1 and r_2. If therefore the response of the organism to the antibiotic cannot be made homogeneous (e.g. by single colony selection), the effects on the zone edge will be minimized when the diffusion time is short and the zone diameter large.

(iv) Factors governing the slope of the dosage-response curve can be evaluated as follows. If d_1 and d_2 are the zone diameters when the amounts of antibiotic in the cup are M_1 and M_2, it is readily shown that

$$d_1 - d_2 = \frac{9{\cdot}21Dt}{R} (\log M_1 - \log M_2), \tag{4}$$

i.e.
$$\frac{d_1 - d_2}{\log M_1 - \log M_2} = \text{‘slope’} = \frac{9{\cdot}21Dt}{R}.$$

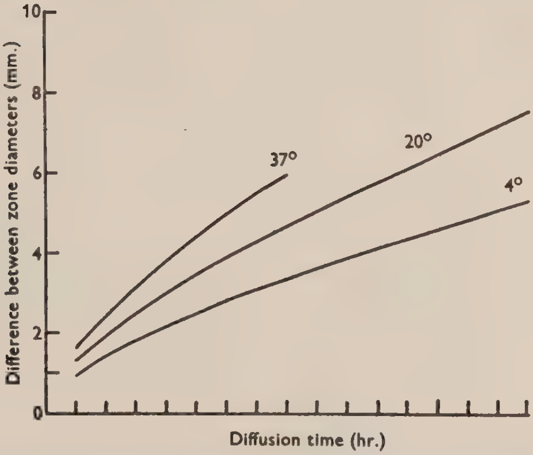

Fig. 5. Theoretical curves demonstrating the relationship between slope and diffusion time for a penicillin assay at levels of 16 and 4 u./ml. Curves are shown for three different temperatures of diffusion. No correction of the 4 and 20° curves is made for subsequent incubation at 37°. The values used for *D* are given in Table 2.

From (4) it appears that the slope will vary directly with diffusion time, and inversely with the mean zone radius. It will also depend directly upon the diffusion constant. Two practical conclusions can be drawn. The first is that when assays of several antibiotics are preformed under standard conditions, and when, as occurs in practice, zone diameters are chosen for convenience to be about the same, the main factor governing the slope will be the diffusion constant. The commoner antibiotics all have similar diffusion constants, and the slopes in their assays will consequently also be similar.

The second conclusion is that the slope can be increased by prolonging the diffusion time and by keeping the antibiotic concentrations and therefore the zone diameters small. In practice, the value of such measures can be limited by loss of definition of the zone edge, for they are precisely opposite to the recommendations of § (iii). Fig. 5, however, illustrates the effect upon the slope of penicillin assays of the length of the diffusion time at different temperatures

The special cases of penicillin and streptomycin assays

When penicillin assays are performed with the I.C.I. strain of *B. subtilis*, and streptomycin assays with *B. subtilis* or other Gram-positive organisms, the zone edges are characteristically very sharp; they do not shift with continued incubation; and contiguous zones do not distort one another. These observations suggested that there might be a sharp drop in antibiotic concentration at the zone edge, which might be due either to its destruction or to its removal by the organisms at that site. Investigations have shown such a drop to occur, although the mechanisms in the two cases are different and must be treated separately.

Penicillin assays

An experiment was devised to discover whether penicillin did in fact diffuse beyond the zone edge of *B. subtilis* seeded plates. For this purpose plates were prepared which were seeded with a mixture of I.C.I. strain *B. subtilis* spores and of *S. lutea.* The sarcina is about three times as sensitive as the subtilis to penicillin, and the two organisms grow well in each other's presence. This point was verified by examination of stained smears from the agar. Beads containing a fixed quantity of penicillin were placed upon plates seeded with the mixture of organisms, and upon control plates containing each of the organisms alone but in other respects identical. Sets of plates were incubated at 28 and 35°, since by varying the temperature it was possible to accelerate or retard the growth of *B. subtilis* relative to that of *S. lutea.* The plates were examined at intervals, and Fig. 6 illustrates diagrammatically the behaviour of the inhibition zones after 7 and 24 hr. The *S. lutea* zones shifted outwards as incubation continued, whereas the *B. subtilis* zones remained fixed. When both organisms grew together the *S. lutea* zones became fixed, and the size at which

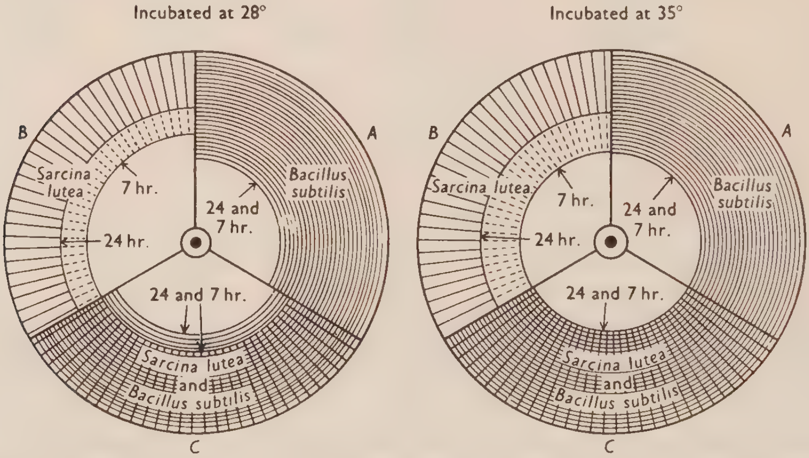

Fig. 6. Scale diagram of an experiment which illustrates the failure of penicillin to diffuse past zone edges formed by *B. subtilis.* Matched plates were poured with agar seeded with *B. subtilis* alone (*A*), *Sarcina lutea* alone (*B*), and a mixture of the two (*C*). Beads containing 16 u./ml. penicillin were placed on each plate and the inhibition zones were measured at intervals during incubation at 28 or 35°. Sarcina alone gave a wide zone which increased with time owing to lysis. In the mixed plate at 28° the Sarcina zone appeared first, but became fixed when the *B. subtilis* zone appeared inside it. At 35° growth of both organisms became apparent at the same time, and Sarcina appeared and remained present right up to the *B. subtilis* zone edge.

they were fixed depended upon the time at which significant growth of *B. subtilis* had occurred. The most probable explanation is that penicillin did not get past the *B. subtilis* zone edge.

Removal of penicillin by adsorption by the organisms might provide a possible explanation of such an effect, but calculations based on the data of Rowley, Cooper, Roberts & Smith (1950) showed that the amount adsorbed would be far too small. We were therefore led to test for penicillinase production. In order that conditions of growth of the *B. subtilis* should be comparable to those obtaining in assays, plates seeded with spores were incubated under standard conditions. After 16 hr. the agar was removed and frozen solid in a closed container, after which it was allowed to thaw. The liquid formed was collected, and was sterilized by filtration through a Gradocol membrane. Aliquots of the liquid were incubated at 37° in the presence of phosphate buffer pH 7·0 for varying periods of time with varying amounts of penicillin. The amount of penicillin destroyed was estimated biologically. In a typical experiment with a filtrate from the I.C.I. strain the rate of inactivation of penicillin, present at an initial concentration of 16 u./ml., was 28 u./ml. filtrate per hour. The enzymic nature of this inactivation was confirmed by showing that the extent was proportional to the time of incubation. Such a low penicillinase activity would escape detection by most methods, but it is sufficient, nevertheless, to destroy penicillin at the zone boundary, as the following calculations show.

Consider an annular ring of agar 25 mm. diam., of depth 4 mm. and of width 0·05 mm. This is approximately the size of the boundary area of a typical inhibition zone, and its volume is 0·015 c.c. When once the organisms have grown, this volume of agar will be able to inactivate approximately 0·42 u. penicillin/hr. Calculation of the amounts of penicillin which would enter such a ring from a bead at the centre containing a solution of penicillin 16 u./ml. showed them to be as follows: after 3 hr., nil; after 7 hr., 0·0174 u.; after 11 hr., 0·067 u.; after 20 hr., 0·19 u. Even if the penicillinase were considerably less active at lower concentrations of penicillin, it would be adequate to inactivate such small amounts.

Although the above considerations suggest that penicillinase production by the test organism will result in sharp zone edges it must be emphasized that only minimal penicillinase production is likely to produce this effect. The Mill Hill strain of *B. pumilus* can be shown to produce more potent or more diffusible penicillinase than the I.C.I. strain, and when it is used the zone edges are somewhat ragged because of small colonies which grow up inside the main zone boundary. Ingram (1951) illustrates well the appearance of scattered colonies inside the inhibition zone with a penicillinase-producing resistant staphylococcus, and remarks that fine crenation of the edge makes it unsuitable for assay work.

Streptomycin assays

We were unable to demonstrate enzymic inactivation of streptomycin or of dihydrostreptomycin by any of the test organisms. It was known, however, from the work of Linz (1949) that Gram-positive organisms adsorb streptomycin, and we therefore studied this adsorption quantitatively. Thick suspensions were prepared of *B. subtilis*, *B. pumilus* and of *Staph. aureus* grown for 16–20 hr. on slopes of assay agar. The suspensions were washed with water, and dried from the frozen state. Varying amounts of the organisms were incubated with varying concentrations of streptomycin in phosphate buffer pH 7·8 for periods ranging from 10 min. to several hours. The organisms were removed by centrifugation and the concentration of streptomycin remaining in the supernatant was estimated biologically (fiducial limits of the assay not more than ±3 % for $P < 0{\cdot}05$). It was found that removal of streptomycin by the organisms was independent of time of contact, but that the amount removed per unit weight of organisms depended upon the concentration of streptomycin present. Fig. 7 illustrates the results of experiments with *Staph. aureus* and *B. subtilis*. The units of streptomycin adsorbed per mg. dry weight of organisms are plotted on a double logarithmic scale against the concentration of streptomycin in u./ml. remaining in the supernatant fluid. It will be seen that in both cases the adsorption fits an adsorption isotherm of the Freundlich type. The equations describing their behaviour are: $x = 20{\cdot}4\ C^{0{\cdot}24}$ for *Staph. aureus* and $x = 1{\cdot}82\ C^{0{\cdot}68}$ for *B. subtilis*, where x = units adsorbed/mg. and C = u./ml. in the supernatant. The greater adsorption by *Staph. aureus* is probably associated with the fact that the suspension used contained much more Gram-positive material than the *B. subtilis*.

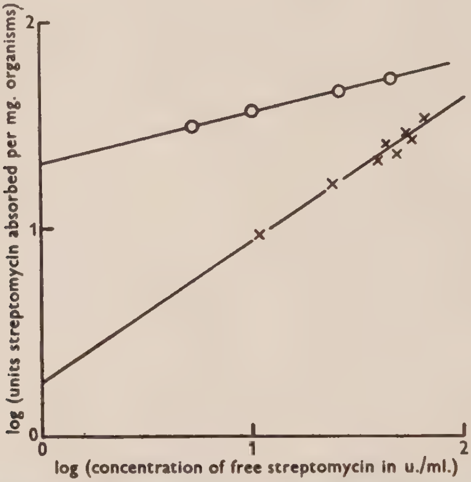

Fig. 7. Relationship between streptomycin adsorbed per mg. dry weight of organisms and concentration of free streptomycin in the medium. Experiments conducted in phosphate buffer pH 7·8, $\mu = 0{\cdot}1$ at 37°. The results are plotted on a logarithmic scale: ○—○, *Staph. aureus*; ×—×, *B. subtilis*.

Adsorption of streptomycin in this way could account for a sharp fall in concentration at the zone edge, as the following illustrative calculation shows.

Let σ be the concentration which would obtain at a given distance from a bead in the absence of adsorption by the organisms, and let C be the actual concentration of free streptomycin in the presence of organisms. $(\sigma - C)$ will be the amount of streptomycin adsorbed by the organisms present in 1 ml. of the agar layer.

The organisms present in 1 ml. after a time t hr. will be $q \,.\, 2^{(t-t^1)/g}$, where q = initial inoculum in mg./ml., t^1 = lag period, g = mean generation time. From the adsorption isotherm we have

$$\frac{\sigma - C}{q \,.\, 2^{(t-t^1)/g}} = aC^b$$

where a and b are constants. We also have, from the diffusion equation,

$$\sigma = \frac{M}{4\pi hDt} \mathrm{e}^{-r^2/4Dt}.$$

Hence
$$\frac{M}{4\pi hDt} \mathrm{e}^{-r^2/4Dt} = C + aq \,.\, 2^{(t-t^1)/g} C^b.$$

From this equation, using the values obtained experimentally, it is possible to calculate the true concentration C at a distance r from the bead after a given time t.

Consider a bead containing streptomycin 80 u./ml. placed upon a cold seeded agar plate which is then incubated at 37°. We know from experience that the zone diameter will be about 2·15 cm. This diameter is, incidentally, almost equal to the calculated value for $D = 0{\cdot}012$ cm.²/hr., critical time 45 hr., and critical concentration 0·03 u./ml. Let us consider then the change of actual concentration with time at a distance $r = 1{\cdot}07$ cm. from the bead, and substitute the following values in the equation: $M = 2$ units (measured), $h = 0{\cdot}4$ cm. (measured), $D = 0{\cdot}012$ cm.²/hr. (estimate based on measurement at 4°), $q = 10^{-4}$ mg. (measured), $t^1 = 1$ hr. (estimated), $g = 0{\cdot}4$ hr. (measured in shaken broth culture), $a = 1{\cdot}82$ (measured), $b = 0{\cdot}68$ (measured).

Calculations give the following results:

Time (hr.)	C = actual concentration (u./ml.)	σ = uncorrected concentration (u./ml.)
1	0·000000005	0·000000005
2	0·00013	0·00013
3	0·0041	0·0043
4	0·018	0·02
5	0·037	0·06
6	0·022	0·11

After 6½ hr., which is equivalent to 13 generations, the density of the organisms in the surface layer would be approximately 2 mg./ml., which is of the order of the maximal growth to be expected, and it is evident that division would cease, and the equation would no longer be applicable. The calculations entail too many approximations to permit exact conclusions, but they show, nevertheless, that once growth begins, at the site which is later to contain the visible zone edge, the adsorption of streptomycin by the organisms already present can, while growth continues, keep the concentration of free streptomycin from significantly exceeding the critical concentration (0·03 u./ml.). In the case of *Staph. aureus*, which adsorbs streptomycin more powerfully, the same argument can be used but the more powerful adsorption will permit the zone diameter to be much smaller—as in practice it is. We have observed, furthermore, that when streptomycin is added to a plate seeded with *B. subtilis* at 38° *after* growth of the test organisms has taken place, no macroscopic lysis occurs. Hence it is immaterial, once the visible zone edge is established, how far streptomycin later penetrates beyond it.

DISCUSSION

To persons experienced in assay procedures many of the conclusions which have been reached above from theoretical reasoning may appear to be matters of common sense. There is some advantage, however, in being able to consider quantitatively some of the factors governing zone diameters. For example it is possible from a knowledge of the zone diameters produced by two different concentrations of a substance, and of the time of diffusion involved, to calculate its diffusion constant. For obvious technical reasons the value obtained in this way may not be very accurate, but it will be obtained with little trouble. It is also an advantage to be aware of the factors concerned in determining the zone edge, since it becomes clear that the ideal of a perfectly sharp zone edge requires either a homogeneous population of test organisms with a sharply defined critical concentration and no tendency to lysis, or the existence of special circumstances such as we have demonstrated in the case of our penicillin and streptomycin assays. In the absence of such circumstances, although of course such devices as layered plates give some improvement, attempts to secure perfect zone edges may lead only to frustration. In the case of aureomycin assays, for example, we have been unable to demonstrate either 'aureomycinase' activity, or adsorption of aureomycin, by our test organisms, and have had to be content with attempts to improve the zone edge by other methods such as the use of a test organism which after growth produces an alkaline reaction, and thereby swings the pH in a direction away from the optimum for aureomycin (Valentine & Johns, 1949).

The prediction that the log. dose will be proportional to the square of the zone diameter, and its experimental verification in most of our assays, may appear to be contrary to common experience, particularly since it should apply to punch plate assays also. This expected relationship would of course become invalid if any factor were to affect small and large zones unequally. As may be seen from Fig. 4, after a given period of diffusion the rate of increase of antibiotic concentration within the zone boundary is greater for the larger amount of antibiotic than for the smaller. The effect of production of a small excess of penicillinase at the zone edge, capable of diffusing inwards, would therefore be to decrease the smaller zones more than the large. Although we have not been able to treat this effect mathematically, it is in the direction which would be required to produce a linear relationship between log. dose and zone diameter, which is that commonly assumed and which we also observed.

From a practical point of view, the distinction between a linear and a quadratic relationship between the log. dose and the zone diameter may prove to be unimportant except when great accuracy is sought. As an example let us consider an assay in which the mean zone diameter for a given dose is 2·00 cm. and for 4× the dose 2·50 cm. If the log. dose is proportional to the diameter, the expected mean diameter for 2× the dose will be 2·25 cm., whereas if the log. dose is proportional to the square of the diameter the expected mean diameter for 2× the dose will be 2·264 cm. In order to distinguish between these two values a variation of 0·014 cm. in the mean diameter for 2× the dose must be significant—i.e. the coefficient of variation must be less than 0·25 %. This condition would require that at least16 replicates be used at each dose level if a single plate were used, and more if several plates were involved.

REFERENCES

Compilation of Regulations for Tests and Methods of Assay and Certification of Antibiotic and Antibiotic-containing Drugs (1951). Vol. I. Washington, D.C., U.S.A.: Federal Security Agency, Food and Drug Administration.

Cooper, K. E. & Woodman, D. (1946). The diffusion of antiseptics through agar gels, with special reference to the agar cup assay method of estimating the activity of penicillin. *J. Path. Bact.* 58, 75.

Friedman, L. & Kraemer, E. O. (1930). The structure of gelatin gels from studies of diffusion. *J. Amer. chem. Soc.* 52, 1295.

Ingram, G. I. C. (1951). Formation of clear zones with 'sensitive' and 'resistant' *Staphylococcus aureus* in penicillin plate assays. *J. gen. Microbiol.* 5, 22.

International Critical Tables (1929). Vol. v, p. 63. New York: McGraw Hill.

Kantorowicz, O. (1951). An antibiotic assay tray. *J. gen. Microbiol.* 5, 357.

Linz, R. (1949). Sur le mécanisme de l'action de la streptomycine. 1. Action de la streptomycine sur les bactéries. *Ann. Inst. Pasteur*, 76, 250.

March, H. W. & Weaver, W. (1928). The diffusion problem for a solid in contact with a stirred liquid. *Physical Rev.* 31, 1072.

Rowley, D., Cooper, P. D., Roberts, P. W. & Smith, E. L. (1950). The site of action of penicillin. I. Uptake of penicillin on bacteria. *Biochem. J.* 46, 157.

Valentine, F. C. O. & Johns, R. G. S. (1949). A suggested method for the titration of certain antibiotics. *Lancet*, ii, 1033.

Vesterdal, J. (1947). Studies on the inhibition zones observed in the agar cup method for penicillin assay. *Acta path. microbiol. scand.* 24, 273.

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LACK, C. H. (1952). *J. gen. Microbiol.* 7, 144

A Simple Hot Box for Microcultures

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SUMMARY: The invention of glass electrothermal heating tape provides a simple method for heating a hot box or incubator without the expense and trouble of a water jacket. An outfit is described for observing microscopically and photographing cultures at controlled temperatures over long periods.

The accompanying photographs illustrate a hot box which we have made in the laboratory out of the wooden case in which the microscope was supplied. Eighteen inches of electrothermal heating tape (Electrothermal Engineering Ltd., supplied by A. Gallenkamp and Co., London), are fixed around the inside of the box; leads from this pass through a Sunvic thermostat, the control of which is on the outside. Two thermometers pass through the sides of the box; they are protected outside by metal shields. A hole is cut in the back of the box and a glass chamber containing water is inserted to allow illumination from outside without heat loss.

In the outfit illustrated here (Pl. 1, fig. 1), a Cooke Universal microscope is shown, with a culture preparation mounted for growth and for observation by phase contrast. There are two sources of illumination at the back of the box: (i) an 8 V. Cooke lamp which slides in and out of the box at the back; (ii) a mercury vapour lamp that is fixed in a well behind the box. For ordinary observation the 8 V. lamp is used, but when more light is required for photography, the 8 V. lamp is taken out and the mercury vapour lamp which is already in proper alinement is used instead. Pl. 1, fig. 2, also shows a Perspex front which is used to help maintain a constant temperature when observations are to be made for a long time.

This hot box has been in continuous use for 9 months and we have had no trouble with it. A little steam and odour come from the thermal tape when first used, but this stops after 2–3 hr. As this tape may be obtained in a range of wattage/foot, it obviously has many laboratory uses.

I wish to thank Mr R. J. Whitley for the photographs.

EXPLANATION OF PLATE

Fig. 1. A microscope in position in its own case showing the thermal tape, the white material on the left.

Fig. 2. Shows the Sunvic control, one thermometer, and the mercury vapour lamp. The plastic screen can be seen in the photograph.

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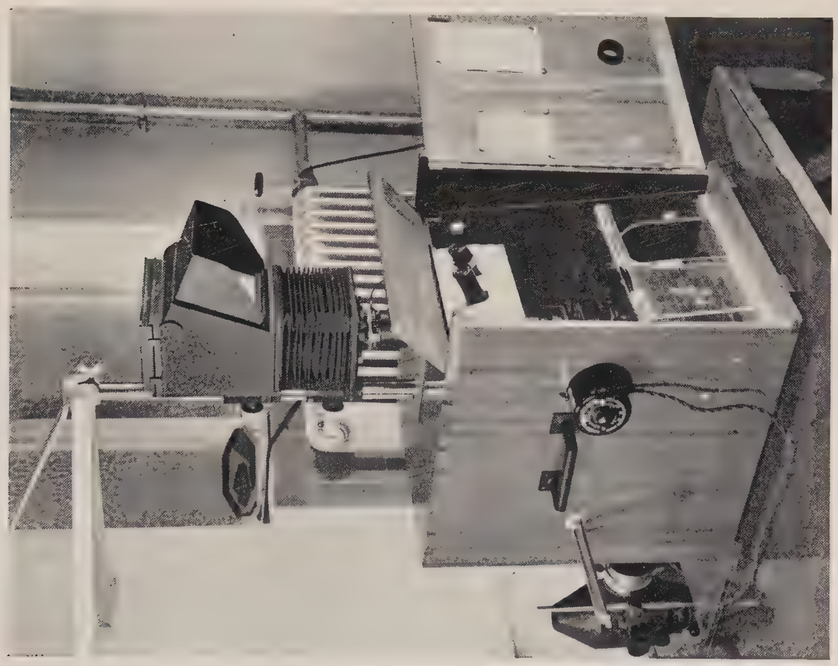

Fig. 2

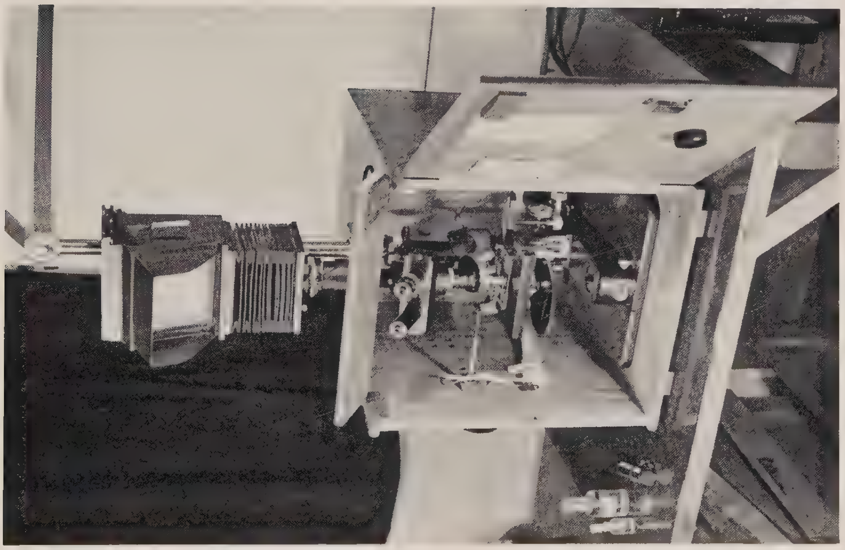

Fig. 1

C. H. Lack—A simple hot box for microcultures. Plate 1

SUGDEN, BRENDA & OXFORD, A. E. (1952). *J. gen. Microbiol.* 7, 145–153

Some Cultural Studies with Holotrich Ciliate Protozoa of the Sheep's Rumen

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SUMMARY: In a suitable buffer at 39° the life of all three species of holotrich ciliates of the sheep's rumen can be extended for one or more days by addition of glucose, fructose, galactose, sucrose, cellobiose, raffinose, inulin, bacterial levan, salicin or melibiose (the least effective). Mannose, glucosamine or galactosamine are definitely toxic in that they greatly shorten protozoan life. This toxic effect is observed even in the presence of glucose. *Isotricha prostoma* and *I. intestinalis*, but not *Dasytricha ruminantium*, will ingest vegetable starch of small grain size, e.g. rice starch, thereby prolonging protozoan life.

The nitrogenous requirements of these protozoa are best met by whole grass juice, which extends the life of the culture for several weeks; and even the ethanolic precipitate from boiled and cleared grass juice is a better nitrogenous supplement than cleared rumen liquor. The ash from this alcoholic precipitate will definitely extend protozoan life in absence of nitrogenous supplement. The effect is probably due to a 'trace' metal, which is not Zn, Fe, Sn, Sr, Mn, Cu or Ni, but may be Ti, Mo, Cr, Co or V.

The holotrich ciliates here studied do not ingest lactobacilli, either when starved or when in an actively motile state after addition of glucose to the medium.

Although certain of the more abundant oligotrich ciliates of the sheep's rumen have been kept alive for long periods *in vitro*, particularly by Hungate (1942, 1943) there is no record in the literature of any attempt to do this with the holotrich ciliates, of which there are usually three species to be found in that situation, viz. *Isotricha prostoma*, *I. intestinalis* and *Dasytricha ruminantium*. The latter species is much the smallest of the three, and occurs in the greatest numbers. All three species are of considerable biochemical interest since it has been shown that they are able, under anaerobic conditions, rapidly to convert certain soluble sugars into a granular starch-like storage polysaccharide which may indeed account for four-fifths of the dry weight of the cell (Oxford, 1951; Masson & Oxford, 1951). In order to study this process more fully we have endeavoured to find out the requirements of these protozoa for growth, multiplication and maintenance for long periods *in vitro*, and now present an account of our positive knowledge on three topics, viz. mineral requirements, carbohydrate requirements and differences in behaviour between the *Isotricha* species on the one hand, and *D. ruminantium* on the other. The nitrogenous requirements of these protozoa have proved to be complex and are not dealt with in any detail in this paper.

METHODS

Animal. Rumen contents were obtained as required from a Cheviot wether (sheep no. 879), aged 4 years, which had been fitted with a permanent rumen fistula in 1950 by Dr A. T. Phillipson of this Institute. During the experiment it was fed on good quality meadow hay and had access to a salt lick.

Basal salt medium for protozoan cultures. The bicarbonate-rich buffer previously used for brief fermentations (Oxford, 1951) was replaced by a more stable, osmotically equivalent liquid of the same pH value (6·8–7·0) based on the formula of Hungate (1942). It contained: NaCl, 0·5; CH_3CO_2Na, 0·15; $NaHCO_3$, 0·1; KH_2PO_4, 0·1; $MgSO_4,7H_2O$, 0·01 %. It was freshly prepared and heated to 39° immediately before use. Later, when the trace element requirements of the protozoa were specifically under investigation, it was enriched by the addition of $CaCl_2,2H_2O$, 0·01 %. All salts used were of Analar quality.

Nitrogenous supplements. Routine experiments with carbohydrate substrates were carried out with an addition of 10 % (v/v) cleared and sterilized rumen liquor to provide nitrogenous nutrients including growth factors. The sterilization was performed by centrifuging strained rumen liquor at high speed ($30{,}000 \times g$) followed by Seitz filtration. Later, after abortive attempts to provide a chemically-defined and soluble nitrogenous substitute for rumen liquor, the latter was replaced with advantage by a 5 % (v/v) supplement of fresh grass juice, prepared as follows.

Nitrogenous and mineral supplements from lawn grass. Freshly cut summer grass, which was free from dicotyledonous weeds, was minced in a domestic meat mincer and strained by hand squeezing in a muslin bag. The juice was then cleared as far as possible by centrifuging, and the supernatant used as the supplement of fresh grass juice referred to above. To prepare an active fraction from this juice it was first boiled for 5 min. to coagulate protein. After recentrifuging and filtration through a Whatman no. 1 paper, the clear liquid, which contained about 1 mg. N (Kjeldahl)/ml., was found to be sufficiently free from bacteria for direct use as a supplement when it was desired to keep bacteria at a minimum. A solid and almost equally efficient fraction was prepared from this liquid by addition of 4 vols. of 95 % ethanol, collection of the precipitate by centrifuging, and drying in a vacuum desiccator, after thorough washing with ethanol on the centrifuge. It contained polysaccharide (levan) and some protein (*c.* 8 % calc. from Kjeldahl N) and was both an adequate carbohydrate source and a fairly complete nitrogenous supplement for the protozoa. A purely mineral and N-free supplement could be prepared from it by simple ignition, when the loss in weight was *c.* 70 %.

Bacterial supplement. Although carefully washed protozoa (see below) were used as inoculum, they were certainly not entirely free from rumen bacteria. After incubation for a day or more *in vitro* in presence of a single soluble carbohydrate, it is quite likely that a markedly different bacterial flora will develop from that which formed the natural environment of the protozoa. It is very difficult indeed to determine by microscopic examination whether or not these protozoa have appreciable numbers of small rumen bacteria inside themselves. This is mainly because of screening by the multitude of storage starch grains (of approximate size $10 \times 3\mu$.) and also because of the presence of numerous mitochondria. It was thought that if a suspension of a large, rod-like bacterium, both facultatively anaerobic and saccharolytic, and hence not likely to be affected unfavourably by rumen conditions, was presented to the protozoa, the ingestion of bacteria by the latter, if it took place, would be plainly visible by vital staining, or even without any staining at all. For this purpose a strain of *Lactobacillus bulgaricus* (NCIB 76) was obtained from the Department of Scientific and Industrial Research, Teddington, Middlesex. Before use it was grown anaerobically for 2 days at 40° on slopes of tomato-glucose agar, and the growth scraped off and suspended in the basal salt medium.

Carbohydrate substrates. All carbohydrates used, save those specifically mentioned below, were of best commercial quality, obtained either from L. Light and Co. or from T. Kerfoot and Co. (Biochemical Reagent quality). The galactose used was glucose-free. Mannose (L. Light) was obtained absolutely ashless by recrystallizing first from redistilled methanol and then from redistilled ethanol. Inulin was a gift from the Edinburgh University Chemical Department, galactosamine hydrochloride was a gift from Prof. M. Stacey, University of Birmingham, and bacterial levan was the freshly prepared specimen used by Masson & Oxford (1951). Glucose-1-phosphate was given by Prof. S. Peat of the University College of North Wales.

Preparation of inoculum. This was done as described by Masson & Oxford (1951, p. 665) under 'carbohydrate fermentations without appreciable bacterial competition'.

Methods of cultivation. These were essentially those described by Masson & Oxford (1951); 100 ml. Erlenmeyer flasks were used, and care was taken to fill them completely with warm buffer, before finally replacing the cork bearing the Bunsen valve. In point of fact, the sheep holotrichs are not nearly as sensitive to atmospheric oxygen as the large rumen oligotrichs, so that the addition of Na_2S, or similar reducing agent, is not only unnecessary but is really disadvantageous in that it tends to prolong the life of the latter. The initial concentration of carbohydrate or other substrate was always about 0·1 %; higher concentrations are liable to cause bursting of cells through excessive starch storage. Cellobiose was used for the positive control rather than glucose because it was used at a more moderate rate by the Protozoa and doubtless by extraneous bacteria also, if any were present. A no-substrate control was also set up for each batch of washed protozoa. Each flask was given a fairly heavy inoculum, representing one-fifth of the total holotrichs separated from 1 l. of rumen contents. Incubation was at 39°.

The culture medium was renewed every 24 hr. after careful decantation of the supernatant. This had the dual purpose of restoring a neutral pH value and of controlling the free bacterial population. On each day microscopic examination was made of two separate small drops of deposit taken from the bottom of the flask, first at 1 hr. or earlier and then at 20 hr., after setting up the culture or replacing the medium, respectively. Starch storage was demonstrated by direct wet staining with Lugol's iodine, after examination for motility, dividing forms, etc. Occasionally counts were made by a simple dilution technique from samples of the well-shaken culture, Lugol's iodine again being used for fixing and staining the cells. Such counts are possible since dead holotrich protozoa are so fragile that they do not remain intact for long. Hence it is safe to assume that nearly all the protozoa counted by this technique were alive before addition of the iodine.

RESULTS

Behaviour of sugars in prolonging protozoan life

With the no-substrate control the course of events was invariably as follows: the starch initially in the cells gradually decreased in amount; the cells never showed any division even during the first 24 hr. but gradually became more and more sluggish before death; the culture died out completely on the third day at the latest. Most of the sugars and other substrates tested did not appreciably affect this behaviour. Such 'neutral' substrates included xylose, arabinose, rhamnose, ribose, fucose, sorbose, maltose, lactose, turanose, trehalose, melezitose, all hexitols, glucuronic and galacturonic acids, glucose-1-phosphate, hexose-diphosphate, dihydroxyacetone, malic, lactic and all lower fatty acids, ethanol, glycogen, soluble starch (as paste), ascorbic acid. Cellulose (in the form of finely divided cotton-wool) was also ineffective.

Table 1. *Carbohydrates which extended the life of sheep holotrichs*

Sugar	Increase in length of life of culture (days)
Glucose	4–5
Fructose	2–3
Galactose	2–3
Sucrose	2
Cellobiose	4–5
Raffinose	2
Inulin	2
Bacterial levan	2
Salicin	3
Melibiose	1

Nitrogenous supplement supplied by addition of 10 % (v/v) cleared and sterilized rumen liquor.

Certain carbohydrates did, however, definitely extend the life of the culture. The course of events was then as follows: increased activity, starch storage and cell division on the first day; little change for the next few days; finally, gradual death of all the cells presumably because no further cell division had occurred.

Table 1 lists the soluble sugars which aided survival of the protozoa.

The different effects of utilizable and non-utilizable substrates are shown clearly by counts made of the population of protozoa 3 days after setting up the experiments (see Table 2).

The 'toxic' sugars. Mannose, glucosamine hydrochloride and galactosamine hydrochloride definitely shortened the life of the culture. With mannose, most cells were abnormally sluggish after only 1 hr. exposure and *D. ruminantium* had completely died within 1 day. There was also a reduced population of the

Isotricha species. Galactosamine, on the other hand, killed all the cells in 1 day. The effect of glucosamine (see also Masson & Oxford, 1951) was greater on the *Isotricha* spp. than on *D. ruminantium.* It killed all the first-named ciliates of both species in 1 hr., whereas some *D. ruminantium* survived overnight. The effect of the amino-sugars was probably not primarily due to the amino group; ethanolamine and *iso*amylamine hydrochlorides had no effect on the protozoa at comparable concentrations. Ammonium chloride was also inert. Essentially the same result was obtained with mannose or glucosamine when an equivalent amount of glucose was added at the same time as the 'toxic' sugar.

Table 2. *Population density of rumen holotrichs after 3 days on various substrates*

	No. cells/ml.	
Substrate	***Isotricha* spp.**	***D. ruminantium***
Cellobiose	760	2040
Galactose	360	1960
Lactose	40	0
Lactate	0	0
None	0	0

Utilization of granular starch by the Protozoa

Both species of *Isotricha* were found to ingest small vegetable starch grains when the latter were added to the rumen liquor basal without soluble sugar. Rice starch was particularly suitable for this purpose. Under these conditions the *Isotricha* spp. lived for 8 days whilst *D. ruminantium* did not ingest any starch grains and died of starvation within 3 days just as if no carbohydrate had been provided.

Neither the *Isotricha* spp. nor *D. ruminantium* were able to utilize purified suspensions of their own storage starch granules, although these granules are on the average much smaller than rice starch grains.

The rates of ingestion and utilization of rice starch grains were determined for protozoa which had been starved for 3 days, as in a no-substrate control, and which were on the point of dying. They were clearly much depleted in storage starch at this point. After addition of the rice starch, samples were withdrawn in 5, 10, 20, 60 and 120 min. and immediately stained with very dilute iodine; the vegetable starch grains then became dark bluish purple and the protozoan storage granules reddish brown. Ingestion of rice starch grains began immediately (see Pl. 1, fig. 1), and within 5 min. an increase in storage granules appeared in the motile cells of the *Isotricha* spp. After 2 hr. these cells contained such a solid mass of iodine-staining material that it was impossible to distinguish between ingested starch and storage starch.

Rate of storage starch deposition with cellobiose as substrate

By means of the technique described above, it was shown that protozoan starch storage with a soluble sugar was a very rapid process also. Within 5 min. some starch had already been laid down, and in less than 1 hr. nearly all the motile cells appeared to be completely filled with starch. With glucose as substrate the effect was even more spectacular.

Grass juice and fractions prepared therefrom as nitrogenous supplements

It is obvious that rumen liquor is not a very satisfactory nitrogenous supplement, since even in presence of a utilizable sugar the cultures never lived more than 8 days. No better results were obtained with any of the usual nitrogenous sources effective for bacteria, such as yeast autolysate, Bacto-peptone, Bacto-tryptone, casein, casein acid hydrolysate, and mixtures of synthetic amino-acids. In fact such supplements, by encouraging bacterial growth, often proved detrimental to the protozoa. On the other hand, centrifuged but unboiled grass juice, added to the extent of 5 % of the volume of the basal medium, gave a most encouraging result in that the culture survived for 1 month, during which time a steady population of organisms of both genera was maintained as frequent divisions kept pace with mortality. When a higher concentration of grass juice was employed (e.g. 10 or 20 %, v/v), the medium was found to contain too much available carbohydrate since bursting of the cells through too rapid starch storage was encountered (see Pl. 1, fig. 2).

A 5 % supplement of boiled juice kept the organisms of all these species alive for 14 days. An advantage of the boiled grass juice was that fewer bacteria developed in the cultures. It was definitely disadvantageous to sterilize the boiled grass juice by Seitz filtration. The results with the boiled juice were regarded as sufficiently encouraging to warrant a further fractionation. The dried ethanol precipitate obtained from it, when added to the medium in 0·3 % concentration, was definitely superior to rumen liquor + cellobiose in that it kept all three species alive for 11 days instead of 7 or 8 days. The result is perhaps even better than it looks since the carbohydrate of the grass juice fraction (a mixture of levans) is probably not as suitable as cellobiose for prolonging protozoan life (see Table 1).

Observations concerning the mineral requirements of the protozoa

Since the ethanol precipitate from grass juice contained carbohydrate rather than protein as the chief organic constituent it readily yielded a clean ash on ignition. This ash, at 0·08 % concentration, was used as a supplement in conjunction with 0·1 % cellobiose. In order to neutralize the alkaline ash it was suspended in water and a little very dilute HCl added, before including in the basal medium. This medium, without any nitrogenous supplement at all, kept all three species alive and active for 8 days, i.e. 4 days longer than in absence of the ash. Very few bacteria developed in these cultures, and the supernatant liquid above the protozoan bottom layer was always quite clear. Since this calcium-rich ash is much more easily obtained than a clean sodium-rich ash from rumen liquor, its composition was regarded as throwing more light on the mineral requirements of the protozoa than did the rumen-liquor ash. Apart from silica, Ca^{++}, Na^{+} and K^{+}, the other metallic constituents, as determined spectrophotometrically at the Macaulay Institute, are listed in Table 3. In the opinion of Dr R. L. Mitchell of that Institute, the tin content is much higher than would be expected for any grass fraction, and most of it has probably been added as a contaminant at some point in the fractionation procedure. The relative concentrations of the other elements are probably normal. A synthetic 'ash', representing a mixture of the first 9 metals in Table 3 with the exception of Ba and Pb, of the following composition was prepared (mg./l. basal medium): $ZnSO_4,7H_2O$, 6·1; $FeSO_4,7H_2O$, 5·1; $SnCl_2,2H_2O$, 1·0; $SrCl_2,6H_2O$, 0·95; $MnSO_4,4H_2O$, 0·6; $CuSO_4$, $5H_2O$, 0·2; $NiSO_4,7H_2O$, 0·2. This was tested in conjunction with cellobiose, but brought about no extension of life of the protozoa, whereas in the control experiment 'natural' ash did in fact extend protozoan life for several days. It is clear, therefore, that a real trace element effect is operative. Any further experiments along these lines would obviously require the rigorous purification of the salts of Na, K, Ca and Mg added to the basal medium.

Table 3. *Trace elements in ash from ethanol precipitate from boiled grass juice*

Element	Concentration in dried ethanol precipitate (p.p.m.)
Zn	460
Fe	340
Sn	180
Sr	104
Mn	52
Ba	40
Pb	40
Cu	20
Ni	15
Ti	6
Mo	5
Cr	2·4
Co	<0·4
V	<0·4

To obtain p.p.m. in the actual culture medium, divide by 333.

Possible ingestion of bacteria by the protozoa

Washed holotrichs which had been 'starved' for 3 days, i.e. kept anaerobically at 39° in the buffer without any addition of carbohydrate, showed no tendency whatever to ingest lactobacilli. The protozoa in this experiment were only sluggishly motile, but no different result was obtained on restoration of motility by addition of 0·01 % glucose together with a copious inoculum of *Lb. bulgaricus*. Apart from examination of the living culture by high dry objective, samples were removed at frequent intervals, the protozoa killed by osmic acid vapour and then examined under phase-contrast oil-immersion (×95) objective. In no instance were any rod-like bacteria seen in the cytoplasm or entering the gullet. This was also confirmed by Gram staining after heat fixation. The protozoan cell as a whole was Gram negative; any Gram positive rods inside it should have been clearly visible.

DISCUSSION

It will be seen that the list of carbohydrates which prolonged the life of the protozoa (Table 1) contains galactose, raffinose and melibiose, in addition to those compounds which Masson & Oxford (1951) found to be detectably converted into storage starch during a 3 hr. fermentation. It is probable that these three sugars are rather more slowly converted to starch than the others. The case of galactose, the only monosaccharide of the three, obviously merits further investigation. With regard to the mechanism of the conversion of soluble monosaccharides to storage starch, our results seem to contain contradictory implications. Thus if glucose-6-phosphate is an intermediate in the starch-forming process the inhibitory effects of glucosamine and mannose are explicable, since these two sugars, as well as glucose and fructose, are phosphorylated by the hexokinase system (see, for example, Long, 1952). On the other hand, the protozoa were not able to make use of glucose-1-phosphate, the assumed immediate precursor, for storage starch formation, when this sugar was dissolved in the medium. Further work is needed to resolve this apparent contradiction.

The uselessness of cellulose for prolonging holotrich life in presence of bacteria seems to dispose of the possibility mentioned by Masson & Oxford (1951) that the sheep holotrichs might utilize cellobiose derived from cellulose by bacterial action in the rumen, for storage starch formation. It now seems more likely, in view of the positive result with salicin, that the β-glucosidase enzyme system, which they undoubtedly possess, is brought into action in their natural environment only to attack the plant glycosides which may sometimes occur in the fodder.

From a biochemical point of view, *Dasytricha ruminantium* is the most bacterium-like of the three holotrich species studied, since it can apparently satisfy its carbohydrate requirements only through a soluble sugar. It is obvious that if the complex soluble nitrogenous requirements of this protozoan are to be worked out in detail, then a careful fractionation of the nitrogenous constituents of grass juice is a necessary preliminary. It is hoped to undertake this when fresh supplies of spring grass are available.

It seems clear that the inorganic requirements of these protozoa are also complex. All that can be said on this point at the moment is that although the ash from a grass juice fraction is beneficial, the effect is not to be ascribed to any of the more abundant metallic ions in this ash, but is probably due to a trace element, possibly Ti, Mo, Cr, Co, V or even to an element not yet detected. Acording to Bowen (1940) vanadium, as $VOCl_2$, aids cell division of the flagellate *Chilomonas paramecium* when added at a concentration up to 10^{-5}M to an inorganic basal medium very similar to that which we have employed. As far as we are aware no work has yet been done on the effect of vanadium on ciliate protozoa.

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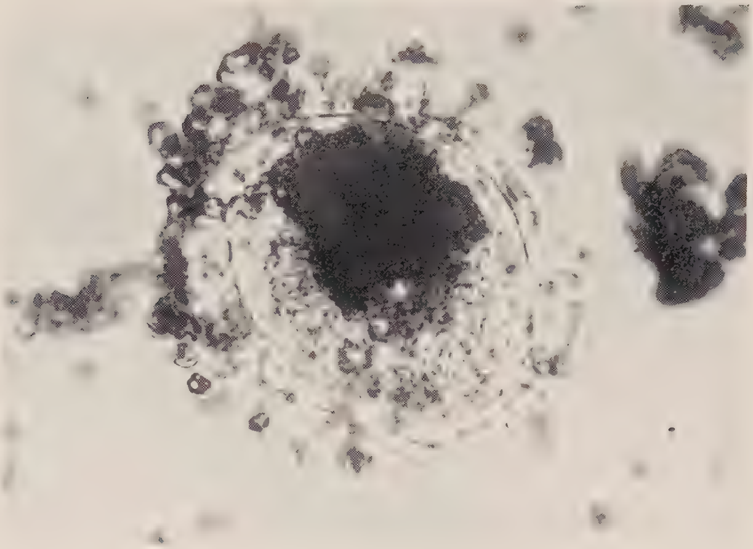

Fig. 1

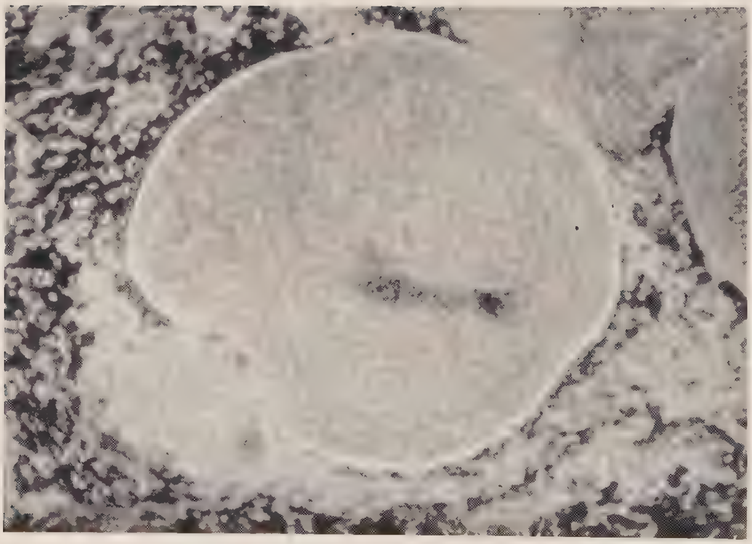

Fig. 2

Brenda Sugden & A. E. Oxford—Culture of rumen holotrich ciliates. Plate 1

galactose we used for the presence of glucose; and particularly to Dr R. L. Mitchell of the Macaulay Institute for Soil Research for the trace element analysis of ash from a grass juice fraction.

REFERENCES

BOWEN, W. J. (1940). Effects of vanadium, copper, manganese on *Chilomonas paramecium*. *Biol. Bull. Woods Hole*, **79**, 114.

HUNGATE, R. E. (1942). The culture of *Eudiplodinium neglectum*, with experiments on the digestion of cellulose. *Biol. Bull. Woods Hole*, **83**, 303.

HUNGATE, R. E. (1943). Further experiments on cellulose digestion by protozoa in the rumen of cattle. *Biol. Bull. Woods Hole*, **84**, 157.

LONG, C. (1952). Studies involving enzymic phosphorylation. 1. The hexokinase activity of rat tissues. *Biochem. J.* **50**, 407.

MASSON, F. M. & OXFORD, A. E. (1951). The action of ciliates of the sheep's rumen upon various water-soluble carbohydrates, including polysaccharides. *J. gen. Microbiol.* **5**, 664.

OXFORD, A. E. (1951). The conversion of certain soluble sugars to a glucosan by holotrich ciliates in the rumen of sheep. *J. gen. Microbiol.* **5**, 83.

EXPLANATION OF PLATE

Fig. 1. Ingestion of rice starch grains by *Isotricha intestinalis*. Small vegetable starch grains can be seen in the gullet. Iodine staining. × 420.

Fig. 2. Bursting of *Isotricha intestinalis* due to excessive storage starch formation in a carbohydrate-rich medium. Clouds of protozoan starch grains are being expelled in two places. Dark ground illumination. × 500.

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The Nature and Behaviour of Inhibitors of Plant Viruses produced by *Trichothecium roseum* Link

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SUMMARY: Culture filtrates of *Trichothecium roseum* contain two heat-stable substances that inhibit infection with plant viruses. One is trichothecin, an antifungal substance with the molecular formula $C_{19}H_{24}O_5$; it sometimes visibly damages bean leaves. The other was isolated as a polysaccharide, $[\alpha]_D^{19^\circ} = -33^\circ$; it contains 1·1–1·4 % nitrogen, yields 60–70 % reducing sugars (as glucose) on acidic hydrolysis, and the predominant (45 %) component sugar is D-galactose. The polysaccharide does not combine with tobacco mosaic virus *in vitro*.

The extent to which infection is inhibited depends on the species of the host plant but not on the identity of the virus. Trichothecin, like ribonuclease, is relatively more effective with beans than with *Nicotiana glutinosa*, whereas the polysaccharide and two derivatives of trichothecin (trichothecolone and acetyltrichothecolone) affect *N. glutinosa* more than beans. Trichothecin inhibits infection when sprayed over leaves a day after they have been inoculated with viruses, but is ineffective when applied 2 days before. The polysaccharide inhibits when sprayed over leaves before inoculation but not after. It is suggested that inhibitors act by temporarily altering the metabolism of leaf cells so that introduced virus particles cannot multiply and are inactivated.

The infectivity of plant virus solutions can be inhibited by extracts from many different kinds of organism. The responsible substances mostly remain unidentified, but it is known that inhibitors can have widely different chemical compositions. For example, among the inhibitors most studied are the two pancreatic enzymes, trypsin (Caldwell, 1933; Stanley, 1934) and ribonuclease (Loring, 1942), a polysaccharide from yeast (Takahashi, 1942, 1946), and a glycoprotein from *Phytolacca esculenta* (Kassanis & Kleczkowski, 1948). Weight for weight, these substances have different activities, but all share some features. They do not obviously damage the host plants; infectivity is inhibited immediately they are mixed with the virus solutions, and it can be restored partly or wholly by diluting the mixtures or removing the inhibitor.

Whether inhibitors act by affecting the viruses or the host plants has been much debated, but remains undecided. Stanley (1934) concluded that trypsin was most likely affecting the plant, but he could find no evidence that the enzyme and virus combined *in vitro*. Not only trypsin (Bawden & Pirie, 1936; Hills & Vinson, 1938; Kleczkowski, 1944) but also the more powerful inhibitors, ribonuclease (Loring, 1942; Kleczkowski, 1946) and the phytolacca glycoprotein (Kassanis & Kleczkowski, 1948), are now known to combine with plant viruses when mixed with them at pH values around neutrality. This provides a ready explanation for inhibition, because the combination might block specific groups on the viruses that need to combine with receptors in the host cell as a first step in infection. However, as Kassanis & Kleczkowski

(1948) pointed out, the results of infectivity tests with mixtures of virus and inhibitor, do not fit a simple quantitative neutralization, such as occurs when toxins and anti-toxins are mixed; in particular, there is no constant ratio of inhibitor to virus that wholly neutralizes infectivity, and the greater the concentration of virus in the inoculum, the smaller is the ratio of inhibitor to virus that is needed to prevent any infections. Thus, although these inhibitors combine with viruses *in vitro*, combination may be incidental to inhibition, which may occur for other reasons. That inhibitors may act on the host plant is suggested by the lack of evidence for any specific action between them and individual viruses, whereas there is some evidence that they may affect different hosts to different extents. For example, in tests with tobacco mosaic virus, trypsin (Stanley, 1934) and ribonuclease (Loring, 1942) more effectively prevented infection of *Phaseolus vulgaris* than of *Nicotiana glutinosa*; also, inhibitors occurring in leaves of some plants prevent the infection of other species but not of the species from which they come (Bhargava, 1951; van der Want, 1951). Such evidence, however, is inconclusive, because different plants might differ in their ability to split non-infective complexes of viruses and inhibitors.

Since Johnson (1938) showed that *Aerobacter aerogenes* and *Aspergillus niger* produce inhibitors of plant viruses, many bacteria and fungi have been found to do so, but only with yeast has there been any attempt to identify the responsible substance (Takahashi, 1942, 1946). Gupta & Price (1950) tested filtrates of forty-nine species of fungi and found that more than three-quarters of them caused some inhibition. Filtrates of *Trichothecium roseum* and *Neurospora sitophila* were the most powerful inhibitors, and experiments with the first led Gupta & Price to conclude that the substance responsible was thermostable and non-dialysable. Their data did not fully substantiate the conclusion that inhibitory power was unaffected by dialysis, for dialysed filtrates inhibited less than others, but the tests were not made in conditions that enabled the significance or the extent of the loss to be estimated accurately. None of their results necessitated the conclusion that only one substance was causing inhibition, and it seemed possible that growth products of different sizes might be concerned.

Trichothecium roseum is antagonistic towards various fungi which are pathogenic to plants (Whetzel, 1909). The substance responsible was isolated and called trichothecin by Freeman & Morrison (1949*a*, *b*), and Freeman & Gill (1950) showed it to be the *iso*crotonyl ester ($C_{19}H_{24}O_5$) of the ketonic alcohol, trichothecolone ($C_{15}H_{20}O_4$). Other metabolic products, rosein I, II and III, were also isolated (Freeman, Morrison & Michael, 1949; Robertson, Smithies & Tittensor, 1949). The work we describe was started to find whether any of these products interfere with the ability of plant viruses to cause infection. Trichothecin does so, but is responsible for only part of the inhibitory power of filtrates, most of which is caused by a polysaccharide, whose isolation and properties are described.

MATERIALS AND METHODS

Infectivity tests were made with viruses and hosts that give countable necrotic local lesions. Most were made with tobacco mosaic virus in *N. glutinosa* L. and the Rothamsted culture of tobacco necrosis virus in French bean (*P. vulgaris* L., var. Prince); some were also made with tobacco mosaic virus in a hybrid tobacco and in *P. vulgaris*, var. Riscardo do Milho, with the tobacco necrosis virus in *N. glutinosa* and the hybrid tobacco, and with tomato bushy stunt virus in *N. glutinosa*. Purified virus preparations were used; those of tobacco mosaic and tomato bushy stunt viruses were made by precipitation methods and of the tobacco necrosis virus by ultracentrifugation. Solutions in water of the substances to be tested were mixed with virus solutions and inoculated to at least six half-leaves. In each experiment, the different treatments were distributed over the test plants in the form of Latin squares or incomplete blocks. Inoculations were made by rubbing half-leaves with the forefinger wetted with the inoculum; the leaves were not washed after inoculation. In some experiments, solutions of the substances were not mixed with the viruses, but were sprayed over whole leaves by means of a glass atomizer.

T. roseum, strain F292, was grown, unless otherwise stated, on the medium described by Freeman & Morrison (1949*a*), which contains 1 % corn steep liquor, 5 % commercial glucose and 0·2 % ammonium tartrate. Undiluted corn steep liquor inhibited infection with the viruses and damaged inoculated leaves, but the complete medium did not. The trichothecin content of filtrates was assayed by measuring the extent to which the germination of spores of *Penicillium digitatum* was inhibited.

Nitrogen was determined by the micro-Kjeldahl method and reducing sugars by Hanes's modification of the Somogyi (1937) method (see Hockenhull & Herbert, 1945). Ribonuclease was estimated by determining the extent to which yeast nucleic acid was rendered unprecipitable by acetic acid after 3 hr. incubation at pH 6·5 and 50° (Kleczkowski, 1946).

EXPERIMENTAL

Inhibition by substances with small molecular weights

Preliminary tests with solutions of crystalline preparations of trichothecin (Freeman & Morrison, 1949*a*) and rosein I, II and III (Freeman, *et al.* 1949) showed that trichothecin at concentrations of 20 mg./l. or higher inhibited infection of French bean by the tobacco necrosis virus and of *N. glutinosa* by tobacco mosaic virus, whereas the roseins had no effect. The extent to which trichothecin decreased the numbers of local lesions varied considerably in different experiments. Usually it inhibited more with tobacco necrosis virus in beans than with tobacco mosaic virus in *N. glutinosa*. On average, in experiments when control inocula produced from 30–70 lesions/half-leaf, trichothecin at 100 mg./l. decreased numbers of lesions to about one-third or a quarter with beans, and to one-half with *N. glutinosa* (Tables 1–3), but the decrease varied considerably in different experiments. The variation was correlated with the physiological condition of the test plants, and, in general. the more susceptible the plants, particularly beans, were to infection, the more trichothecin decreased the numbers of infections. At concentrations of 50 mg./l. or higher, trichothecin often caused obvious damage to bean leaves, but it rarely did so to *N. glutinosa* even at 200 mg./l. Gupta & Price (1950) found that undiluted filtrates from cultures of *T. roseum* 'caused visible burning of the leaves', an effect that seems attributable solely to the trichothecin present. Solutions of crystalline trichothecin caused the same damage as culture filtrates of the same trichothecin content, and filtrates from which trichothecin was removed caused none. In our tests with beans, trichothecin at 100 mg./l. sometimes had no macroscopic effects, sometimes caused scattered necrotic spots, and sometimes caused extensive necrosis, areas of 1 sq.cm. or more collapsing. Except that no lesions developed in such collapsed areas, the extent to which leaves were damaged and infection was inhibited were not obviously correlated. The number of lesions was often much decreased in bean leaves which showed little or no injury.

Table 1. *Effect of trichothecin and its derivatives on infection*

	Mean no. of lesions/leaf in		
	N. glutinosa with		
Substance	Tobacco mosaic virus	Tomato bushy stunt virus	*P. vulgaris* with tobacco necrosis virus
Trichothecin	70	40	22
Trichothecolone	32	24	50
Acetyltrichothecolone	3	3	62
*iso*Trichothecolone	131	60	52
Crotonic acid	112	56	43
*iso*Crotonic acid	108	53	70
Water (control)	132	50	69

Inoculations were made with substances at 100 mg./l. and the viruses at 2·5 mg./l.

In addition to tests with metabolic products of *T. roseum*, some were also made with derivatives of trichothecin (Freeman & Gill, 1950). The results of one series of tests are given in Table 1, which shows that trichothecolone and acetyltrichothecolone inhibited infection. In sharp contrast to trichothecin, both these more effectively prevented infection of *N. glutinosa* than of beans. Both substances, but the acetyl derivative more strikingly, also showed a reverse action to that of trichothecin, in causing damage to *N. glutinosa* leaves but not to bean leaves. Thus the introduction of different groups into these substances can entirely change their behaviour towards different plants, and there is a clear suggestion that ability to inhibit infection by viruses may be correlated with ability to interfere with the metabolism of the host plant.

Inhibition with non-dialysable substances

The fact that trichothecin is not the only substance in culture filtrates of *T. roseum* that inhibits infection with plant viruses was readily demonstrated by comparing the inhibition caused by untreated filtrates with that caused by dialysed filtrates and by water solutions of trichothecin. Table 2 shows the summarized results of tests with a filtrate that initially had a total solid content of 30 g./l., of which 130 mg./l. was trichothecin. After dialysis, for 1 day against tap-water and 1 day against distilled water, the trichothecin content, assayed biologically, was less than 1 mg./l., and the total solid content was 2·5 g./l. The crude filtrate was a much stronger inhibitor than water solutions of equal trichothecin content, as also was the dialysed filtrate in tests with *N. glutinosa*. When trichothecin was added to the dialysed filtrate, in an amount equal to that present in the original filtrate, the original inhibitory and leaf-damaging actions were fully restored, suggesting that trichothecin is the only dialysable product of the fungus with these actions.

Table 2. *Inhibiting effects of trichothecin and dialysed filtrate*

	No. of lesions/leaf with		
Inhibitor	Tobacco mosaic virus in *N. glutinosa*	Tobacco necrosis virus in *N. glutinosa*	Tobacco necrosis virus in beans
Culture filtrate 10/13	1	1	10
Dialysed filtrate 10/13	2	4	20
Trichothecin 100 mg./l.	45	23	27
Dialysed filtrate 10/13 Trichothecin 100 mg./l.	1	2	9
Water (control)	155	101	78

Inhibitors were mixed with equal volumes of virus solutions; tobacco mosaic virus at 5 mg./l., tobacco necrosis at 2 mg./l. for test in beans and 10 mg./l. for test in *N. glutinosa*.

Table 2 shows that removing trichothecin from the filtrate had little effect on the extent to which infection of *N. glutinosa* was inhibited, and that the dialysed filtrate was relatively more effective in decreasing numbers of infections in this host than in beans. The similar results from tests with tobacco mosaic and tobacco necrosis viruses in *N. glutinosa* suggest that the identity of the host plant rather than that of the virus determines the extent to which infection is inhibited. That this is so, has been confirmed repeatedly. When mixed with trichothecin or dialysed filtrate and inoculated to *N. glutinosa*, tomato bushy stunt virus gives results that closely parallel those with tobacco mosaic and tobacco necrosis virus in this host. In beans, tobacco mosaic virus behaves like tobacco necrosis virus; that is to say, trichothecin is relatively more effective in inhibiting infection than it is in *N. glutinosa*, whereas the dialysed filtrate is relatively less effective. In tests with the dialysed filtrate and hybrid tobacco plants, similar results are obtained with both tobacco mosaic and tobacco necrosis viruses, and inhibition is intermediate between that obtained with *N. glutinosa* and beans.

Gupta & Price (1950) found that infection of beans by southern bean mosaic virus was inhibited when leaves were rubbed with *T. roseum* culture filtrate 30 min. after inoculation with the virus. We have made no tests in which leaves were rubbed twice, as this damages the leaves and itself decreases numbers of infections, but have made many in which whole leaves were sprayed with solutions at different times before and after they were inoculated with viruses. These showed that infection can be inhibited when the delay between inoculating with virus and applying culture filtrate is much more than 30 min., and that trichothecin and the non-dialysable component behave very differently when applied at intervals before and after inoculating with virus. Some of the differences are illustrated in Tables 3 and 4. When sprayed on the leaves 2 or 3 days before they are inoculated, trichothecin causes little or no inhibition; sprayed a day before, it decreases the numbers of lesions, although less so than when sprayed up to 1 day after inoculation. By contrast, the non-dialysable component has little or no effect when sprayed on the leaves an hour after inoculation, whereas all the pre-inoculation sprayings inhibit infection, the effect decreasing only slowly with increasing delay between spraying and inoculation.

Table 3. *Effect of spraying bean leaves with trichothecin and dialysed filtrate before and after inoculation with tobacco necrosis virus*

Time of spraying	No. of lesions/leaf when sprayed with Trichothecin (100 mg./l.)	Dialysed filtrate
3 days before inoculation	290	150
2 days before inoculation	230	140
1 day before inoculation	118	120
Unsprayed	300	245
1 hr. after inoculation	33	234
4 hr. after inoculation	51	240
1 day after inoculation	82	285

Table 4. *Effect of spraying* N. glutinosa *leaves with trichothecin and dialysed filtrate before and after inoculation with tobacco mosaic virus*

Time of spraying	No. of lesions/leaf when sprayed with Trichothecin (100 mg./l.)	Dialysed filtrate
1 day before inoculation	26	11
1 hr. before inoculation	33	2
Unsprayed	70	54
1 hr. after inoculation	26	44
4 hr. after inoculation	33	52
24 hr. after inoculation	35	61

The different behaviour of the two inhibitors was further shown when solutions were sprayed over either the upper or lower surfaces of leaves which were inoculated on their upper surfaces an hour before or after the sprayings. In tests with *N. glutinosa* and tobacco mosaic virus, dialysed filtrate inhibited strongly only when the upper surfaces were sprayed before inoculation, whereas with beans and the tobacco necrosis virus all four sprayings with trichothecin inhibited infections equally strongly. Such differences in behaviour of the two inhibitors are most likely attributable simply to differences in their size. The small molecules of trichothecin can presumably penetrate uninjured leaves and diffuse readily from cell to cell, whereas the non-dialysable inhibitor, like a virus, needs a wound through which to enter. Within an hour of being rubbed with water, leaves recover sufficiently from their injuries to prevent infection when they are sprayed with virus solutions (Kalmus & Kassanis, 1945). This rapid healing probably explains the short period after inoculation during which spraying with the dialysed filtrate inhibits infection. Its relatively lasting effect when applied before inoculation suggests that it persists unchanged on the sprayed leaves until they are rubbed and that such substances might be economically useful as preventive sprays.

Of more interest in understanding the mechanism of inhibition is the transitory effect of trichothecin when applied before inoculation. It seems that, on entering cells, it changes them in such a manner that infection with viruses is prevented. The changes are temporary and, after a day or so, the cells return to normal, at least in their susceptibility to infection with viruses. Trichothecin decreases the number of infections when it is applied a day after leaves have been inoculated with tobacco necrosis virus. As visible lesions appear in 2–3 days after inoculation, it seems that the changes caused by trichothecin are such that, even in cells where infection is already established, the virus can still be prevented from multiplying sufficiently to cause a lesion.

Gupta & Price (1950) found that boiled culture filtrates of *T. roseum* inhibited infection and concluded that the inhibitor was not a protein. However, as ribonuclease resists boiling at pH 6, this conclusion is not necessarily valid. The need for further evidence was emphasized by the fact that the first culture filtrate we studied depolymerized yeast nucleic acid. Comparisons with solutions of pancreatic ribonuclease suggested that the enzyme content was too small to account for more than a little of the inhibitory power, and showed that ribonuclease behaved differently from dialysed filtrate in that it inhibited infection of beans much more strongly than infection of *N. glutinosa* (Table 5). Further differences between the two were also demonstrated by boiling at different pH values. Boiling for 10 min. at either pH 6 or 8 leaves the inhibitory power of the dialysed filtrate unimpaired; indeed, in many tests it was slightly enhanced, suggesting that heating may have destroyed some component that interferes with its action. Boiling at pH 8 destroys ribonuclease activity (Kleczkowski, 1946) and largely destroys its ability to inhibit infection of *N. glutinosa*, the host more sensitive to dialysed filtrate; ability to inhibit infection of beans, the host less sensitive to dialysed filtrate, is lessened but not destroyed (Table 5).

These results virtually excluded the possibility that the dialysed filtrates inhibit because they contain a protein resembling pancreatic ribonuclease. For the ribonuclease in the filtrate to be responsible, it would need to differ from pancreatic ribonuclease in its resistance to boiling and its relative action

Table 5. *Effect of boiling on inhibitory power of dialysed filtrate and ribonuclease*

	No. of lesions/leaf with		
Solution	Tobacco necrosis virus in beans	Tobacco mosaic virus in *N. glutinosa*	Tomato bushy stunt virus in *N. glutinosa*
Dialysed filtrate 1/2	32	7	5
Boiled dialysed filtrate 1/2	24	6	2
Ribonuclease 0·01 %	0·5	8	7
Boiled ribonuclease 0·01 %	20	112	153
M/30 Phosphate buffer	85	150	208

Dialysed filtrate and pancreatic ribonuclease were boiled for 10 min. at pH 8 in M/30 phosphate buffer, and brought to pH 7 before mixing with virus solutions at 5 mg./l.

towards beans and *N. glutinosa*. That it is not responsible was shown by later work with other batches of culture filtrates and, more particularly, with fractions from them, which inhibited infectivity strongly but had no detectable ribonuclease activity and probably contained no protein.

Purification of the non-dialysable inhibitor

As a preliminary to attempting the isolation of the non-dialysable inhibitor, the growth of *T. roseum* was studied on media containing fewer initial components with large molecules than that used for trichothecin production. Instead of 50 g. commercial glucose/l. as used by Freeman & Morrison (1949*a*), 40 g. pure glucose/l. were used, and instead of 10 ml. corn steep liquor/l. a range from 10 to 0 ml. was tried, and compared with 10 ml./l. of the diffusate fraction from dialysed corn steep liquor. *T. roseum* grew slowly for the first 14 days on the medium containing no corn steep liquor, but on the others growth resembled that on the usual medium. At the end of 28 days the trichothecin content varied from 66 mg./l. in the culture on medium containing 10 ml. corn steep liquor/l. to 18 mg./l. for the medium free from corn steep liquor. All the culture filtrates strongly inhibited infection of *N. glutinosa* by tobacco mosaic virus, showing that the non-dialysable inhibitor was produced in satisfactory amounts in media containing pure glucose and diffusate from corn steep liquor; the culture on medium free from corn steep liquor was the least effective inhibitor, probably because of its slow growth.

The first fractionation was made with 7 l. of culture filtrate harvested after *T. roseum* had grown for 28 days on a medium containing 1 % (v/v) of diffusate from dialysed corn steep liquor. The filtrate, which contained 42 mg. trichothecin/l., was concentrated to 500 ml. by evaporation *in vacuo*, and then dialysed for 1 day against tap water and 1 day against distilled water. After removing a small precipitate by filtration, the fluid was concentrated to 100 ml. by further evaporation *in vacuo*. After removing a little insoluble material by centrifugation, the solid content of the fluid was 42·4 g./l.; the trichothecin content was 37 mg./l., showing that 99 % of that originally present was removed by dialysis. Infectivity tests with tobacco mosaic virus in *N. glutinosa* showed that little of the non-dialysable inhibitor was lost during these treatments.

The concentrated dialysed filtrate had an apparent glucose content of 1·1 g./l. before hydrolysis and of 25·5 and 26·5 g./l. after hydrolysis with 2N-H_2SO_4 for 2 and 4 hr. respectively. The addition of an equal volume of 10 % trichloroacetic acid separated a precipitate, equal to 1·2 g. from a litre. This material caused little inhibition and the inhibitory power of the filtrate was only slightly decreased by its removal. The addition of 5 vols. of ethanol precipitated crude polysaccharide amounting to 31 g./l. of concentrated filtrate. This precipitate contained the bulk of the inhibitor, though some remained in the ethanolic mother liquor, which also still contained some carbohydrate.

These preliminary tests showed that the inhibitor could be dried *in vacuo* and redissolved without losing activity, and suggested that it was associated with polysaccharide, a major component of the filtrate, and not with the minor protein component. This was further suggested by tests in which the concentrated filtrate was treated according to the Sevag method for removing protein. After removing a little insoluble material (fraction B27/29*a*), the filtrate was twice shaken with chloroform (Sevag, Lackman & Smolens, 1938). A little chloroform gel was produced, which was freed from chloroform and extracted with 50 ml. water (B27/29*b*). The aqueous supernatant fluid from the chloroform treatment was then fractionally precipitated with increasing strengths of ethanol to yield the five polysaccharide fractions B27/29 (1)–(5), and the ethanolic mother liquor B27/29 (6). A little glacial acetic acid was added during the precipitation to facilitate flocculation.

The yields and properties of the fractions are shown in Table 6. The different fractions differ in nitrogen contents and amounts of reducing sugars yielded by hydrolysis. If it can be accepted that the chloroform treatment removed all protein, it can be assumed that the polysaccharide contains nitrogen and that infectivity is inhibited by polysaccharide. The fraction precipitated by 50 % (w/w) ethanol is, weight for weight, less active as an inhibitor than the others, but the fractions do not separate sharply into those that inhibit infectivity and those that do not.

Similar results were obtained with two other batches of culture filtrate, one of 13 l. from a medium containing no corn steep liquor and one of 15 l. from a medium containing 1 % (v/v) of diffusate from dialysed corn steep liquor. After concentration by evaporation *in vacuo*, dialysis and twice shaking with chloroform, precipitation with 80 % (w/w) ethanol gave precipitates of crude polysaccharide amounting to 0·27 and 0·54 g./l. of initial culture filtrate, the mean yield for the three isolations being 0·41 g./l. The protein fractions separated by the treatment with chloroform accounted for less than 5 % of the inhibitory power, which can be attributed to polysaccharide occluded in the protein-chloroform gel. The bulk of the activity was in the precipitate separated by ethanol, but some remained in the ethanolic mother liquor, which also contained polysaccharide.

The crude polysaccharide was precipitated with 80 % ethanol and redissolved in water five times, and then treated with decolorizing charcoal and filtered. When treated with increasing strengths of ethanol between 50 and 80 % (w/w), the polysaccharide again produced a series of fractions all of which inhibited infectivity, contained from 1·1 to 1·8 % nitrogen, and yielded from 50 to 70 % reducing sugars after acid hydrolysis. Again, the fraction precipitated by 50 % (w/w) ethanol was the least effective inhibitor and had the highest nitrogen content. The more active fractions precipitated in the range 60–75 % (w/w) ethanol contained between 1·1 and 1·5 % nitrogen and yielded 60–70 % apparent glucose after acid hydrolysis.

Table 6. *Properties of polysaccharide fractions*

Fraction	Fraction precipitated at ethanol concentration (%, w/w)	Yield of fraction (g.)*	Kjeldahl nitrogen (%)	Reducing value as glucose after hydrolysis for 3 hr. with 2 N sulphuric acid at 100° (%)	No. of lesions per leaf with tobacco mosaic virus in *N. glutinosa*
Insoluble residue, B27/29*a*	—	0·080	—	—	—
Ethanolic fractions:					
B27/29 (1)	50	0·198	2·4	49·6	10
B27/29 (2)	60	0·617	1·5	59·9	1·6
B27/29 (3)	66	0·438	1·2	63·6	1·3
B27/29 (4)	75	0·247	0·9	53·7	0·6
B27/29 (5)	80	0·058	1·7	48·5	1·3
B27/29 (6) (Mother liquor)	—	0·441	—	—	2
'Protein' B27/29*b*	—	Trace	—	—	10
Water (control)	—	—	—	—	97

* Total recovery = 2·079 g. (88 % of starting material).
Separation of fractions described in text.
For infectivity test virus solution at 5 mg./l. mixed with equal volume of solutions containing 1 mg./ml. of substance, except for B27/29*b*, which was diluted 1/5.

The similarity in behaviour of the polysaccharides separated from the different batches of culture filtrate suggests that there was no gross heterogeneity in the preparations, but there is insufficient evidence to show whether they consist of a single form or of a mixture of similar though not identical types. It seems likely that a variety of different polysaccharides can inhibit infection with plant viruses, and that the inhibitors produced by many fungi and bacteria are polysaccharides. The polysaccharide obtained from yeast by Takahashi (1946) seems to differ from that produced by *T. roseum*. Similarly, other polysaccharides from a species of *Rhizobium*, supplied to us by Dr A. Kleczkowski, and from several species of basidiomycetous fungi, supplied to us by Mr M. V. Tracey, have all inhibited infection of *N. glutinosa* by tobacco mosaic virus.

Nature of component sugars of the polysaccharide inhibitor

The polysaccharide fractions precipitated by 60–75 % (w/w) ethanol were almost colourless powders which dissolved readily in water to give clear pale yellow solutions at concentrations of 20 g./l. Specific rotation was:

$$[\alpha]_D^{19^\circ} = -33^\circ\ (c=2 \text{ in } 2\,\text{N sulphuric acid}).$$

Partition chromatograms were made with neutralized hydrolysates containing 11·5 g./l. reducing sugars estimated as glucose. Filter papers were irrigated with a mixture of *n*-butanol, ethanol, water and ammonia (45, 5, 49 (v/v) and 1 % (w/v) respectively), D-Galactose, D-glucose and D-fructose were used as reference sugars. The chromatograms gave a well-defined spot corresponding to D-galactose and a rather weak spot with an R_F value of 0·098. The polysaccharide gave no reaction with Seliwanoff's reagent, showing that it contains no appreciable quantities of ketose sugars. Uronic acids were not detected in the polysaccharide (20 mg.) by Tollens's naphthoresorcinol test.

One gram of polysaccharide was hydrolysed by boiling for 3 hr. in 2 N-H_2SO_4, freed from sulphate with baryta and made up to 50 ml. with water. The solution had a copper-reducing value calculated as glucose of 1·154 g./100 ml. On this basis the mean rotation of the component sugars, $[\alpha]_D^{18^\circ}$, was +86·6°. (Equilibrium value for D-galactose is +81°.) The reducing value of the hydrolysate calculated as glucose corresponded to 57·7 % of the polysaccharide.

The neutralized hydrolysate (15 ml.) was evaporated *in vacuo* to a syrup (about 0·5 ml.) and treated with glacial acetic acid (5 ml.). The clear solution was seeded with a trace of D-galactose. After standing at 0° for a few days a crystalline deposit of D-galactose (0·090 g. = 30 % of the original polysaccharide) was present, m.p. 162°; mixed melting point with authentic D-galactose (m.p. 164°) was 162°.

D-Galactose was determined in the hydrolysate by the method of Hirst, Jones & Woods (1947). The neutralized hydrolysate (10 ml.) gave D-galactose methylphenylhydrazone (0·1146 g.) corresponding to 45·0 % D-galactose in the original polysaccharide. After recrystallization from pyridine/ethanol (50/50, v/v) mixture, the crystalline product melted at 186°; mixed melting point with authentic D-galactose methylphenylhydrazone (m.p. 188°) was 187°.

Crude polysaccharide (1·0 g.) in water (35 ml.) was oxidized with concentrated nitric acid (sp.gr. 1·42; 15 ml.) and gave a crystalline deposit of mucic acid (0·2722 g.), m.p. 206°. After recrystallization the product melted at 216° and its melting point was not depressed on admixture with authentic mucic acid (m.p. 216°).

Tests for combination between polysaccharide and virus

Infectivity tests with solutions containing mixtures of tobacco mosaic virus and the polysaccharide from *T. roseum* give results similar to those when the virus is mixed with pancreatic ribonuclease or the phytolacca glycoprotein. That is to say, there is no fixed ratio of inhibitor to virus that wholly neutralizes infectivity; the ratio of polysaccharide to virus needed decreases with increasing virus concentration, and concentrated non-infective mixtures become infective when diluted. With ribonuclease and the phytolacca glycoprotein this phenomenon can be attributed to dilution causing non-infective complexes of virus and inhibitor to dissociate, because combination between the virus and these substances *in vitro* is clearly shown by the separation of paracrystalline precipitates from appropriate mixtures.

There is no visible indication that the *T. roseum* polysaccharide combines with tobacco mosaic virus, and mixtures of the two at widely different ratios, in the presence and absence of salts, have never precipitated. This is no proof that the two fail to combine, for they could form a soluble complex, like that formed when trypsin is mixed with the virus (Kleczkowski, 1944). Evidence that they do not combine was obtained by experiments in which mixtures of virus and polysaccharide were centrifuged at 40,000 r.p.m. for 1 hr. Three solutions were centrifuged: (i) contained tobacco mosaic virus at 1 mg./ml.; (ii) contained polysaccharide at 1 mg./ml.; (iii) contained a mixture of the two at this concentration. Nothing sedimented from the solution of polysaccharide (i), whereas the other two gave birefringent pellets of similar sizes and appearances. After washing with water, the two pellets were dissolved in water, when analyses for carbohydrate and nitrogen showed them both to have the usual values for the virus. Similarly, the supernatant fluid from the centrifuged mixture had the same carbohydrate content as the solution that contained only the polysaccharide, and the two were also equally effective in preventing infection of *N. glutinosa* by tobacco mosaic virus and of beans by tobacco necrosis virus.

We have made no similar tests to demonstrate that trichothecin does not form a soluble complex with tobacco mosaic virus, but its ability to inhibit infections when applied to leaves a day after inoculation with virus seems to render unnecessary any suggestion that inhibition depends on combination between the two.

DISCUSSION

The main thing that calls for discussion is the mechanism whereby infection is inhibited. Several of our results bear on this problem; they permit the possibilities to be better assessed than previously and suggest a general hypothesis for the action of inhibitors. There seems little doubt that inhibitors directly affect the host plants rather than the viruses. This is strongly implied by the demonstration that the relative efficiency of different inhibitors is determined by the species of the host plant and not by the identity of the virus. It becomes an almost inescapable conclusion from the demonstration that substances which do not combine with viruses *in vitro* can inhibit infection, for this renders unnecessary any suggestion that hosts differ in behaviour because they differ in their ability to release infective virus from non-infective complexes of virus and inhibitor.

With inhibitors like ribonuclease and the phytolacca glycoprotein, which do combine with the viruses, it is impossible to show that this combination plays no part in preventing infection. They may act in a different manner from the inhibitors produced by *T. roseum*, but there are so many similarities between the behaviour of different inhibitors that this seems unlikely. Many substances that are not good inhibitors of infectivity also combine with viruses *in vitro*. These, like ribonuclease and the phytolacca glycoprotein, are oppositely charged from the viruses at pH values around 6 (Kleczkowski, 1946), and combination more likely occurs because of this than because of any specific affinity between these substances and viruses. The combination, therefore, is probably irrelevant to inhibition of infectivity, and it has been unfortunate that the inhibitors previously studied do combine with viruses. There is no reason to assume that the inhibitors from *T. roseum* are unique in failing to combine. The inhibitor from yeast also seems not to. Although Takahashi (1946) concluded that it probably inhibits because of 'a reaction involving the inactivator and some group in the virus particle which is necessary for its infectivity', there is no evidence that the two do combine and his electrophoretic measurements suggest that they do not, for the mobility of the virus was unaffected by the presence of the inhibitor.

By analogy with the specific adsorption of bacteriophages on to susceptible bacteria, and from the fact that washing leaves immediately after they are inoculated with viruses does not prevent infection, it is reasonably assumed that a first step in initiating infection is for virus particles to combine with some specific receptors in the host cells. Inhibitors previously studied have been applied simultaneously with, or immediately after, the virus, and their effects have been considered mainly as preventing this postulated first step. They might do this in one of two ways, either by combining with the virus particles or with the essential receptors. The possibility that different types of sites might be concerned in combination does something to account for the fact that substances with widely different compositions act as inhibitors of infectivity, but is a barely adequate explanation. As trichothecin inhibits infection when it is sprayed over leaves a day after they have been inoculated with viruses, there is clearly no reason to assume that inhibitors are substances that interfere only with the first step and act because they have a greater affinity either for viruses or their receptor sites than these have for one another. Instead, it seems more reasonable to postulate that inhibitors are substances whose presence so alters the physiology of the host cells that these no longer support virus multiplication. This view calls for no common attributes among inhibitors, except that they should differ from normal components of cells they affect and, because of this, stimulate the cells to some unusual activity. It adequately explains the fact that extracts from leaves of some plants prevent infection of some other species but not of themselves (Bhargava, 1951; van der Want, 1951).

Trichothecin and its two derivatives, trichothecolone and acetyltrichothecolone, clearly affect the metabolism of plants in which they strongly inhibit infection, for they sometimes cause macroscopic lesions. The failure of these, or other, inhibitors to promote obvious changes in host plants, is no reason to assume they have had no effect; it may mean only that changes in metabolism have proceeded less far or have been more promptly or completely counteracted. The effects of inhibitors resemble those caused by exposing plants to atmospheres containing 50 % carbon dioxide (Kalmus & Kassanis, 1944), or to irradiation with ultraviolet light (Bawden & Kleczkowski, 1952). Both these treatments make bean leaves temporarily refractory to infection with tobacco necrosis virus; in some but not all conditions both treatments also obviously damage the leaves. All such treatments are most reasonably interpreted as acting by diverting the cell metabolism in directions that preclude the customary diversion of protein synthesis to virus formation that follows the introduction of virus particles. During the period of changed metabolism conditions seem such that introduced virus particles are destroyed or rendered non-infective, for although leaves treated with trichothecin, carbon dioxide or ultraviolet light, after a time regain their initial susceptibility to infection, virus introduced during the refractory period does not multiply.

There is no positive evidence for this unitarian hypothesis of inhibitor action, but it covers the facts as now known more simply and adequately than any other. In putting it forward, there is no intention of suggesting that all inhibitors produce the same type of metabolic change in host cells. There is much evidence to show that the readiness with which viruses infect differs greatly with changes in the physiological state of the host. Deviations from the normal metabolism of the host in any of many possible ways might equally upset the sensitive balance necessary for infection and virus multiplication to occur.

REFERENCES

Bawden, F. C. & Kleczkowski, A. (1952). Ultra-violet injury to higher plants counteracted by visible light. *Nature, Lond.* 169, 90.

Bawden, F. C. & Pirie, N. W. (1936). Experiments on the chemical behaviour of potato virus X. *Brit. J. exp. Path.* 17, 64.

Bhargava, K. S. (1951). Some properties of four strains of cucumber mosaic virus. *Ann. appl. Biol.* 38, 377.

Caldwell, J. (1933). The physiology of virus diseases in plants. *Ann. appl. Biol.* 20, 100.

Freeman, G. G. & Gill, J. E. (1950). Alkaline hydrolysis of trichothecin. *Nature, Lond.* 166, 698.

Freeman, G. G. & Morrison, R. I. (1949*a*). The isolation and chemical properties of trichothecin, an antifungal substance from *Trichothecium roseum* Link. *Biochem. J.* 44, 1.

Freeman, G. G. & Morrison, R. I. (1949*b*). Some biological properties of trichothecin, an antifungal substance from *Trichothecium roseum* Link. *J. gen. Microbiol.* 3, 60.

Freeman, G. G., Morrison, R. I. & Michael, S. E. (1949). Metabolic products of *Trichothecium roseum* Link. *Biochem. J.* 42, 191.

Gupta, B. M. & Price, W. C. (1950). Production of plant virus inhibitors by fungi. *Phytopathology*, 40, 642.

Hills, C. H. & Vinson, C. G. (1938). Particle size of tobacco mosaic virus. *Res. Bull. Mo. agric. Exp. Sta.* no. 286.

Hirst, E. L., Jones, J. K. N. & Woods, E. A. (1947). The quantitative determination of galactose, mannose, arabinose and rhamnose. *J. chem. Soc.* Pt. II, p.1048.

Hockenhull, D. J. D. & Herbert, D. (1945). The amylase and maltase of *Clostridium acetobutylicum*. *Biochem. J.* 39, 102.

JOHNSON, J. (1938). Plant virus inhibitors produced by micro-organisms. *Science*, **88**, 552.

KALMUS, H. & KASSANIS, B. (1944). Reduction by carbon dioxide of susceptibility of beans to tobacco necrosis viruses. *Nature, Lond.* **154**, 641.

KALMUS, H. & KASSANIS, B. (1945). The use of abrasives in the transmission of plant viruses. *Ann. appl. Biol.* **32**, 230.

KASSANIS, B. & KLECZKOWSKI, A. (1948). The isolation and some properties of a virus-inhibiting protein from *Phytolacca esculenta*. *J. gen. Microbiol.* **2**, 143.

KLECZKOWSKI, A. (1944). Combination of potato virus X and tobacco mosaic virus with pepsin and trypsin. *Biochem. J.* **38**, 160.

KLECZKOWSKI, A. (1946). Combination between different proteins and between proteins and yeast nucleic acid. *Biochem. J.* **40**, 677.

LORING, H. S. (1942). The reversible inactivation of tobacco mosaic virus by crystalline ribonuclease. *J. gen. Physiol.* **25**, 497.

ROBERTSON, A., SMITHIES, W. R. & TITTENSOR, E. (1949). The chemistry of fungi IV. Rosenonolactone from *Trichothecium roseum* Link. *J. chem. Soc.* Pt. II p. 879.

SEVAG, M. G., LACKMAN, D. B. & SMOLENS, J. (1938). The isolation of the components of streptococcal nucleoproteins in serologically active form. *J. biol. Chem.* **124**, 425.

SOMOGYI, M. (1937). A reagent for the copper-iodometric determination of very small amounts of sugar. *J. biol. Chem.* **117**, 771.

STANLEY, W. M. (1934). Chemical studies on the virus of tobacco mosaic. I. Some effects of trypsin. *Phytopathology*, **24**, 1055.

TAKAHASHI, W. N. (1942). A virus inactivator from yeast. *Science*, **95**, 586.

TAKAHASHI, W. N. (1946). Properties of a virus inactivator from yeast. *Science*, **104**, 377.

WANT, J. P. H. van der (1951). Investigations on carnation mosaic. *Tijdschr. PlZiekt*, **57**, 72.

WHETZEL, H. H. (1909). A fungus living as a parasite upon another fungus. *Ont. nat. Sci. Bull.* **5**, 3.

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ADDENDUM

Since submitting our paper for publication we have seen another on the subject by Gupta & Price (*Phytopathology*, 1952, **42**, 45), in which they conclude, as we do, that filtrates of *T. roseum* inhibit infection by altering host-plant susceptibility and not by acting directly on virus particles. Some of our experiments resemble theirs and the results are generally similar. Apparent discrepancies occur because they used unfractionated filtrates, which were assumed to contain only one inhibitory substance. They describe differences between the sensitivity of certain host plants, but differences between the behaviour of beans and *N. glutinosa* are obscured because tests were made with mixtures of trichothecin and the polysaccharide inhibitor. They also report inhibition of infection in the upper surfaces of bean leaves when the lower surfaces were sprayed with filtrate, an effect for which our results show trichothecin is solely responsible.

EVANS, D. G. & ADAMS, MARY O. (1952). *J. gen. Microbiol.* 7, 169–174

The Inability of the Capsular Material of *Haemophilus pertussis* to produce Protective Antisera

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SUMMARY: Bacterial suspensions of capsulated *Haemophilus pertussis*, prepared from cultures freshly isolated from infected mice, or from Phase I strains maintained on Bordet Gengou medium, produced in rabbits antisera which contained specific agglutinating and complement-fixing antibodies and which protected mice against experimental pertussis infection. Organisms which had been washed three times with distilled water and freed from capsular material also produced similar antibodies. More than one antigen may be involved in the production of these antibodies.

On the other hand, bacilli-free preparations, containing the capsular material in a concentrated form, produced antisera with only low levels of agglutinating, complement-fixing and protective antibodies.

The experiments indicated that the antigens of *H. pertussis* which produced these antibodies are somatic rather than capsular.

It was previously shown (Evans, 1944) that pertussis antisera, from rabbits immunized with a heat-killed suspension or a detoxified extract of bacilli, protected mice against the infection induced by the intranasal instillation of *Haemophilus pertussis*. The protective action of the antisera was associated mainly with their specific agglutinating property, for antisera from which the agglutinins had been absorbed with heat-killed whole bacilli had a greatly reduced protective action. The ease with which absorption occurred and the completeness of the effect, suggested that the specific antigen involved was situated at the surface of the bacillus and was responsible for the production of protective antibody. It has been demonstrated that *H. pertussis* is surrounded by a capsular substance (Lawson, 1940) or slime layer (Klieneberger-Nobel, 1948). We have therefore investigated what part this substance might play in the production of protective antibody by comparing antisera prepared against whole capsulated bacilli, bacilli from which the capsular material had been removed, and capsular material in a concentrated form free from bacilli.

EXPERIMENTAL

Staining of capsular material

The method employed by Lawson (1940) was used. It involved the fixing of dry smears, made from cultures on Bordet Gengou medium (BG), by phosphomolybdic acid, washing with water, then with methanol and staining first with a concentrated solution and later with a dilute solution of Wright's stain in glycerol. The organisms stained red and appeared embedded in an amorphous capsular material which stained sky-blue. Sometimes, in thin smears, isolated organisms were seen surrounded by capsular material, the boundary of which was not well defined. Often, capsular material was seen in irregular masses completely detached from the bacilli. No capsular material was seen in smears prepared from organisms which had been treated with water before fixing. Ninety strains of *H. pertussis* freshly isolated from whooping cough cases were examined, and each showed the presence of capsular material. Organisms cultured from mice which had been infected either intranasally (Burnet & Timmins, 1937) or intracerebrally (Kendrick, Eldering, Dixon & Misner, 1947) appeared similar on staining to those freshly isolated from cases of whooping cough.

Six strains which had been subcultured for several months on BG had capsular material, although it was decidedly less in quantity and not as deeply stained as with freshly isolated strains. Capsular material was also seen with two avirulent strains of *H. pertussis*, but the material was extremely diffuse, only faintly stained and did not appear in large masses.

From many observations it was considered that the capsular material was present in the most well-defined state and in the greatest quantity in those strains of *H. pertussis* which had been freshly isolated either from cases of whooping-cough or from infected mice.

Capsular material could not be demonstrated by Lawson's method in ten strains of *H. parapertussis* or in several strains of *Bacterium coli* and *Staphylococcus aureus*.

Concentration of capsular material and preparation of suspensions

In preparing concentrated capsular material, organisms from experimentally infected mice were cultured directly on BG; these cultures appeared to possess the greatest quantity of capsular material. It was difficult to obtain pure primary cultures from the lungs of mice infected intranasally, but when the intracerebral route was used, cultures from infected brains were invariably pure.

The following procedure was adopted. Twenty to thirty mice were infected intracerebrally with *H. pertussis* from a 24 hr. growth on BG suspended in Hartley broth. The infecting dose was such that the majority of the mice might be expected to die about the 10th day. On the 7th day after inoculation, mice were killed; those dying before that time were discarded. The brains of the mice were removed aseptically, frozen at -80° and ground aseptically with carborondum. The resulting paste was suspended in broth and left to stand for 1 hr. to allow gross particles to settle. The supernatant fluid was then centrifuged at 10,000 r.p.m. for 30 min. and the deposit suspended in a small volume of broth. The brains were ground in batches of five and with the extract from one batch 10 plates of BG medium were inoculated so as to obtain a confluent growth. A total of 50–60 plates were inoculated each time. After 48 hr. incubation the growth was scraped off with a loop and suspended in a small volume of sterile water. This suspension was designated MB/BG/U; organisms cultured from Mouse Brain on Bordet Gengou and Untreated. Part of this suspension was dried from the frozen state and preserved.

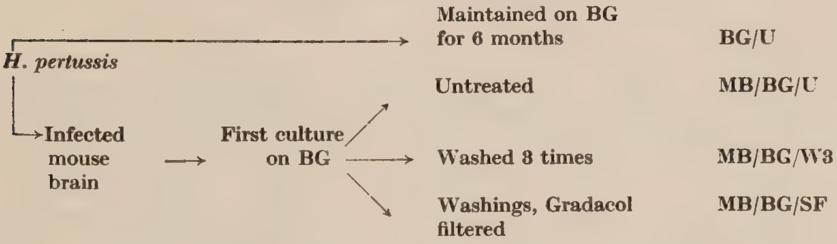
The rest of the suspension was diluted with sterile distilled water to give an opacity equal to no. 4 Brown's tube, transferred to a round-bottom flask and gently shaken for 1 hr. in a mechanical shaker. The suspension was then centrifuged at 10,000 r.p.m. for 30 min. when the water-clear supernatant was removed and kept for further use. The deposit was resuspended in an equivalent volume of water, reshaken for 1 hr., then centrifuged and the supernatant discarded. The deposit was resuspended in water and shaken a third time, centrifuged again, the supernatant discarded and the thrice-washed organisms dried from the frozen state and preserved. The preparation was designated MB/BG/W3; organisms cultured from Mouse Brain on Bordet Gengou and Washed 3 times.

The water-clear supernatant from the first washing was filtered through a Gradacol membrane of 850 mμ, A.P.D. and the filtrate dried from the frozen state. The dry material was dissolved in 5 ml. of distilled water and distributed into ampoules in 1 ml. amounts and redried. Thus, the washings from the confluent growth of *H. pertussis* on 50–60 plates were concentrated in 5 ampoules. This material was designated MB/BG/SF; Supernatant Fluid from washed organisms cultured from Mouse Brain on Bordet Gengou.

An adequate amount of the two preparations MB/BG/U and MB/BG/W3 was obtained from two batches of infected mice, but in preparing MB/BG/SF, many batches of mice were used on account of the low yield of material.

A fourth type of preparation was also made from strains which had been subcultured on BG every 2 or 3 days for 6 months. At the end of this period each strain, which was still agglutinated to titre by Phase I serum, was grown on BG for 48 hr. and the growth scraped off with a loop into distilled water and dried. This preparation was designated BG/U; organisms cultured on BG and Untreated.

The above experiments thus gave four different preparations:



Preparation of antisera

Antisera were prepared in rabbits against each of the above preparations. The various strains used are shown in Table 1.

Suspensions of either washed or unwashed bacteria were prepared for inoculation by suspending the dried bacteria in saline to give an opacity equal to no. 6 Brown's tube. Rabbits were inoculated first subcutaneously and later intravenously with increasing doses over a period of approximately 3 months, the total inoculum amounting to 5–10 mg. dry bacteria.

Each rabbit receiving the MB/BG/SF preparations was given a longer series of injections and received subcutaneously each week for 4 months the dried material from one ampoule dissolved in saline. Thus each dose contained the concentrated washings from the growth on 10 plates of BG, the total amount inoculated being derived from 150–200 plates. Expressing the dose in terms of dry bacteria, each rabbit received the concentrated washings from 250–300 mg. of dry bacteria.

Tests with sera from inoculated rabbits

Each rabbit was bled at the end of its course of injections and the sera were tested for agglutinins, complement-fixing antibodies, antitoxin, and their ability to protect mice against pertussis infection.

Agglutination tests. These were made with a suspension of living *H. pertussis* from a 48 hr. growth on BG (opacity = no. 4 Brown's tube). Incubation of suspension and serum dilutions was for 2 hr. at 37° and readings were finally made after the tubes had remained at room temperature overnight.

Complement-fixation tests. These were made with a thiomersalate-killed suspension of *H. pertussis* diluted to 1/5 opacity of no. 4 Brown's tube, 3 M.H.D. of complement and 3 % sheep cells sensitized with 5 M.H.D. of haemolysin. Fixation was for 1 hr. at 37°, and after adding the haemolytic system there was further incubation at 37° for 1 hr., when the tests were read. Titres for both agglutination and complement-fixation tests are given in Table 1 as reciprocals.

Antitoxin tests. A saline suspension of living *H. pertussis* from a 48 hr. growth on BG was prepared to contain 20–40 minimal skin reacting doses of toxin in 0·2 ml. Falling two-fold dilutions of this suspension were mixed with an equal quantity of undiluted serum. The mixtures were left at room temperature for 1 hr. before they were injected intradermally in rabbits. By this method it was possible to detect whether 0·1 ml. of undiluted serum would neutralize the smallest dose of toxin which gave a skin reaction. Rabbits were observed for 1 week.

Protection tests. Mice were used in groups of *c.* 20 and each mouse received intraperitoneally 0·5 ml. of serum diluted 1/2. The mice were inoculated intranasally, 3–4 hr. after receiving serum, with approximately 10–100 minimal infecting doses of a suspension of *H. pertussis* from a 24 hr. culture. At least three tests were made at the same time, and always included one serum prepared against either washed or unwashed bacteria, one prepared against concentrated washings, and normal rabbit serum. Occasionally some of the mice died, but the majority remained alive and were killed 14 days after infection when the lungs were examined and cultured by smearing a cut surface over half a BG plate. With each animal, the degree of severity of the lung lesions and the extent of growth of *H. pertussis* were recorded. Mice which failed to yield growth from the lung were considered to have been protected, and these mice usually did not have lung lesions.

RESULTS

The results of the *in vitro* serological tests are given in Table 1. The agglutination titres of the sera prepared against the three kinds of bacterial suspension, BG/U, MB/BG/U and MB/BG/W3, ranged from 3000 to 20,000. Significant deductions cannot be made from differences in titre of the three kinds of serum, as relatively few rabbits were immunized, but the two sera in the MB/BG/W3 group each showed a lower titre than those in the BG/U and MB/BG/U groups. Sera prepared against MB/BG/SF, however, showed decidedly lower agglutination titres, from <20 to 320. Similarly, the complement-fixing titres of the sera prepared against the bacterial suspensions were higher (16 to 128) than those prepared against the concentrated washings (<2 to 16). Antitoxin was not detected in any of the sera.

Table 1. In vivo *and* in vitro *properties of pertussis antisera*

Preparation used for immunizing rabbits	Strains of *H. pertussis* from which the preparation was made	No. of rabbit	Agglutination titre	Complement-fixation titre	No. of mice protected/no. inoculated in each test	Total proportion protected		Total % protected	
BG/U	A1	1	20,000	128	12/20, 10/15, 16/19	38/54	105/133	70·4	79·0
	A18	2	10,000	128	18/20, 20/20, 15/19	53/59		89·8	
	A10	3	10,000	16	14/20	14/20		70·0	
MB/BG/U	A1	4	10,000	16	14/19	14/19	61/77	73·7	79·3
	18–323	5	20,000	128	14/18, 15/20, 18/20	47/58		81·0	
MB/BG/W3	A1	6	3,000	64	17/19	17/19	32/39	89·4	82·0
	18–323	7	5,000	32	15/20	15/20		75·0	
MB/BG/SF	A1	8	320	8	7/19, 9/17, 4/20	20/56	38/154	35·7	24·7
	A1	9	160	16	7/20	7/20		35·0	
	A1	10	20	16	5/20	5/20		25·0	
	18–323	11	<20	<2	1/20	1/20		5·0	
	18–323	12	160	2	2/20	0/18		0·0	
	18–323	13	<20	<2	5/20	5/20		25·0	
None	—	14	<20	<2	4/19, 0/15, 3/20	7/54	22/182	13·0	12·1
	—	15	<20	<2	1/17, 3/17, 8/18	12/52		23·1	
	—	16	<20	<2	0/20, 0/20, 0/16	0/56		0·0	
	—	17	<20	<2	3/20	3/20		15·0	

BG/U = Organisms grown on BG and suspended in saline.
MB/BG/U = Organisms from infected mouse brain, grown on BG and suspended in saline.
MB/BG/W3 = Organisms prepared as with MB/BG/U but washed three times with water.
MB/BG/SF = Concentrated washings from organisms prepared as with MB/BG/U.

The results of the protection experiments are also given in Table 1 and indicate the proportion of mice protected against infection. Table 2 gives the degree of infection in the unprotected animals as indicated by lung lesions and growth of *H. pertussis*. Antisera prepared against the bacterial suspensions, either washed or unwashed, either from mouse brain cultures or from cultures which had not been passaged through mice, gave protection. The percentage of mice protected with each of the three types of antisera were similar, 79, 79·3 and 82. On the other hand, antisera prepared against the concentrated washings of the bacilli gave decidedly less protection, the average percentage of mice protected by the six sera being 24·7. Two of these sera gave negligible or no protection, two others were no better than one of the normal sera and the two which gave the best protection were greatly inferior to the least potent serum made against bacterial suspensions. The degree of severity of the lung lesions and the extent of the growth of *H. pertussis* from lung cultures also indicated the low protective action of the MB/BG/SF sera compared with that of sera prepared against the bacterial suspensions.

Table 2. *The protective properties of pertussis antisera shown by (a) severity of lung lesions and (b) extent of growth of* H. pertussis *from lung*

Serum	Total no. of mice treated	No. and (%) of mice showing varying degrees of severity of lung lesions				No. and (%) of mice giving varying degrees of growth of *H. pertussis*			
		***	**	*	0	+ + +	+ +	+	−
BG/U	133	7	5	7	104	3	13	12	105
		(5·2)	(3·8)	(12·8)	(78·2)	(2·3)	(9·8)	(9·0)	(78·9)
MB/BG/U	77	0	1	15	61	1	7	8	61
		(0)	(1·3)	(19·5)	(79·2)	(1·3)	(9·1)	(10·4)	(79·2)
MB/BG/W3	39	2	4	1	32	0	2	5	32
		(5·1)	(10·2)	(2·6)	(82·1)	(0)	(5·1)	(12·8)	(82·1)
BM/BG/SF	154	91	17	12	34	55	36	25	38
		(59·1)	(11·0)	(7·8)	(22·1)	(35·7)	(23·4)	(16·2)	(24·7)
None	182	108	32	23	19	92	47	21	22
		(59·3)	(17·6)	(12·6)	(10·4)	(50·5)	(25·8)	(11·5)	(12·1)

Macroscopic lung lesions: 0, normal lung; *, **, ***, increasing degrees of severity of lung congestion. Extent of growth of *H. pertussis* from lung: −, no colonies; +, < 100 colonies; + +, numerous colonies; + + +, confluent growth.

CONCLUSIONS

It is evident that the antigens of *H. pertussis* which produce agglutinating, complement-fixing and protective antibodies are somatic rather than capsular. It is probable that the antibodies detected in the antisera against the washings from *H. pertussis* were a result of the small amount of somatic antigen removed from the bacilli during washing rather than to the capsule itself.

REFERENCES

Burnet, F. M. & Timmins, C. (1937). Experimental infection with *Haemophilus pertussis* in the mouse by intranasal inoculation. *Brit. J. exp. Path.* **18**, 83.

Evans, D. G. (1944). The protective properties of pertussis antisera in experimental infection. *J. Path. Bact.* **56**, 49.

Kendrick, P. L., Eldering, G., Dixon, M. K. & Misner, J. (1947). Mouse protection tests in the study of pertussis vaccines. *Amer. J. publ. Hlth*, **37**, 803.

Klieneberger-Nobel, E. (1948). Capsules and mucoid envelopes of bacteria. *J. Hyg., Camb.* **46**, 345.

Lawson, G. M. (1940). Modified technique for staining capsules of *Haemophilus pertussis*. *J. Lab. clin. Med.* **25**, 435.

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A Simple and Inexpensive Microforge

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SUMMARY: A microforge is necessary for fashioning the glass tools used in micromanipulation. A simple method of improvising one from a microscope, an eye-cautery and a micromanipulator is described. Its design is based on that of the de Fonbrune microforge. It is capable of the same movements, and the methods of working described for that instrument can be followed.

A number of different types of micromanipulator are commercially available, but the glass tools needed for their use are not, and have to be fashioned by means of a microforge for the particular purpose in view. Methods for forging microinstruments have been described clearly and in great detail by de Fonbrune (1949). His descriptions apply to his own microforge, which is a highly versatile instrument, simple to handle and giving reproducible results. Unfortunately it may not be available to every worker needing microtools. The apparatus described here, which was designed to make tools for a Singer high-power micromanipulator (Barer & Saunders-Singer, 1948), reproduces the main features of the de Fonbrune microforge at a very low cost. Only one part has to be specially made and the machining involved is simple; the rest of the equipment is readily available.

Microforges have been designed by other workers with similar objects in view: cheapness, versatility and reliability. Bretey & Browaeys (1944) described a simple piece of equipment to be attached to a microscope stage or to one of a pair of micromanipulators. In their instrument the principle of traction by a weight was employed with the pull applied horizontally; the microtool was heated by means of a fine cautery mounted on a micromanipulator. They suggested that all the essential techniques of more expensive microforges could be carried out with their apparatus. A more complicated instrument was described by Thaysen & Morris (1947); it consisted of a gas forge for preliminary work and an electric forge for the completion of microtools. The tool was mounted on one micromanipulator and the electric forge on another; the principle of traction by weight was not used.

The apparatus and its use

The de Fonbrune microforge consists essentially of a low-power microscope in the horizontal position, fitted with an angle ocular, a rotating mechanical stage, and built-in substage light. The mechanical stage carries a clip which can be turned on an axis perpendicular to the stage, and locked at any point in its rotation. Thus, by the combined effect of rotating the stage and the clip, a microtool attached to the latter can be set within the optical field at any angle in a vertical plane parallel to the stage. A swivelled mounting on the left of the microscope frame carries the heating element and air-blast pipes. The movement of the heating element in the vertical and horizontal planes within the optical field is governed by fine-adjustment controls. The whole forge is fixed on a base which contains the controls which regulate lighting, the strength of the electric current for the heating element, and the strength of the air-blast.

A Zeiss microscope fitted with a rotating mechanical stage and a Beck angle binocular was in use in this laboratory, and it was realized that if a clip capable of carrying a microtool could be fitted to the stage of this instrument, an exact functional replica of the optical and mechanical systems of the de Fonbrune machine would be achieved. A simple adapter for this purpose was therefore designed (Fig. 1 and Pl. 1, fig. 1). It consists of a flat strip of plated brass having at one end the clip (1) for the microtool and at the other a key (2) which fits the groove of the mechanical stage. The clip (1) is a modification of the Singer clamp (Registered Design No. 847073). It has a friction bearing with the face of the strip, and is able to rotate completely in one plane with a freedom of movement governed by the tightness of the spring-loaded nut (3). The key (2) is designed to hold the adapter parallel to the microscope stage, and far enough away from it to give clearance between the stage and the nut (3); the adapter is locked into the groove of the stage by the screw (4). This arrangement also has the effect of setting the plane of the microtool sufficiently clear of the mechanical stage to give traction weights an unimpeded

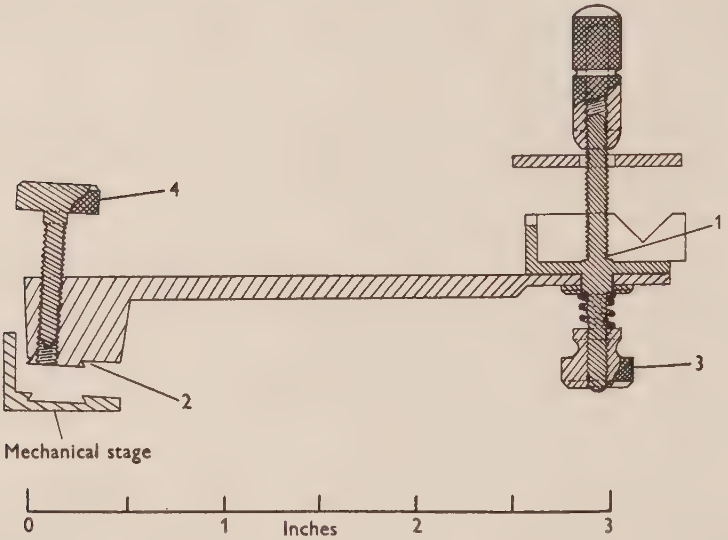

Fig. 1. Sectional drawing of the adapter. A section of the microscope mechanical stage is included to show how the key (2) of the adapter fits into the keyway which normally carries the right-hand clip of the stage. (1) The clip which holds the microtool. (2) The key, which is designed to fit the keyway of the mechanical stage. (3) A spring-loaded nut which controls the rotation of the microtool clip. (4) Locking screw, by means of which the adapter is fixed in position.

vertical drop. The adapter is, in fact, nothing but an extension of the mechanical stage made in such a way that it holds the microtool away from the stage and allows it to be rotated at its point of attachment.

The heating element for the microforge is an ordinary surgical eye-cautery mounted in a lightweight cautery handle (9 cm. long and 1 cm. in diameter). Such handles contain a small switch operated by finger pressure; with the microforge this switch is not used and must be strapped firmly with tape in the 'on' position. The cautery handle is mounted on the micromanipulator to give the fine control needed during the process of forging. Current is provided by a 'cautery box' (output 4 V., 24 amp.) for both the heating element and the substage lamp. Built-in illumination is convenient but may be adequately replaced by an ordinary microscope lamp. The eye-cautery assembly and box are readily available commercially.

The apparatus is set up for use as illustrated (Pl. 1, fig. 2). The microscope is used in the horizontal position; if necessary it may be clipped to a baseboard for greater stability. When the controls for the mechanical stage of the microscope are on the right, the cautery box is placed on that side, and the micromanipulator on the other: for 'left-handed' microscopes this arrangement must be reversed. The adapter is mounted at the right of the mechanical stage and locked into position. The cautery is attached to the micromanipulator so that the cautery handle and the plane of the 'V' of the heating element are horizontal and level with the optical axis of the microscope. A 1 in. low-power objective is used, and the binocular, fitted with low-power eye-pieces (× 6), is swung up into the position shown. An angle attachment of this sort, whether monocular or binocular, is very convenient but not indispensable. The rheostats on the cautery box are set to zero and the box is connected to the cautery and substage lamp by their respective flexes.

Preliminary work is carried out on the microtool by hand as described by de Fonbrune, and it is then clipped on to the adapter. It is adjusted by hand until the tip of the tool appears in the field of view of the microscope when brought into focus. Similarly, the micromanipulator is cautiously moved by hand under naked-eye observation until the tip of the cautery is almost touching the end of the microtool, after which its movements are governed by the controls of the micromanipulator, under vision through the microscope. From this point onwards de Fonbrune's instructions can be followed with little modification.

The design makes no provision for an air-blast. If the microforge is used in conjunction with a Singer micromanipulator, there would be no difficulty in attaching a pipe of suitable shape to the tool rod of this machine, just below the clamp holding the cautery, and connecting it by a flexible lead to a supply of air under pressure. The pipe and cautery would then move together as one unit, an arrangement similar to that found in the de Fonbrune microforge. However, for most of the common microforge techniques an air-blast is not essential.

It should be emphasized that the principal advantage of a microforge of this design is that it is not a permanent piece of equipment for a single purpose only. It can be assembled in a minute or two, and, after use, when the adapter has been taken off, the microscope is ready for micromanipulation or for any other use.

My thanks are due to Mr I. M. Macdonald-Murray for carrying out machining and for technical advice.

REFERENCES

Barer, R. & Saunders-Singer, A. E. (1948). A new single-control micro-manipulator. *Quart. J. micr. Sci.* **89**, 439.

Bretey, J. & Browaeys, J. (1944). Confection des instruments au moyen des micromanipulateurs auxquels ils sont destinés. *C.R. Soc. Biol., Paris,* **138**, 465.

de Fonbrune, P. (1949). *Technique de micromanipulation.* Paris: Masson et Cie.

Thaysen, A. C. & Morris, A. R. (1947). The preparation of microtools for the micromanipulator. *J. gen. Microbiol.* **1**, 221.

EXPLANATION OF PLATE

Fig. 1. The mechanical stage with the microscope drawtube removed, showing the adapter in position. A partly fashioned microtool is mounted in the clip of the adapter.

Fig. 2. A general view of the complete assembly. The Singer micromanipulator is on the left, carrying the eye-cautery. The microscope in the centre has a partly processed microtool in the same optical plane as the tip of the cautery, and carries a substage light: at this angle the latter is hidden behind the stage. The cautery box controlling the current to the cautery and substage lamp is on the right.

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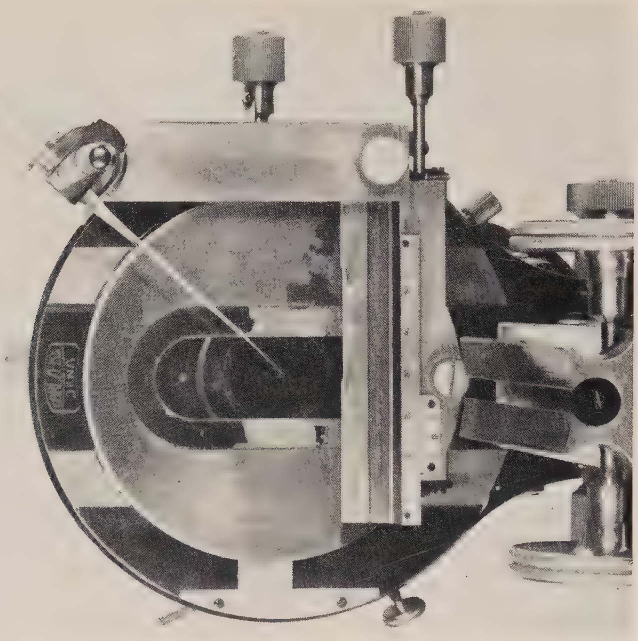

Fig. 1

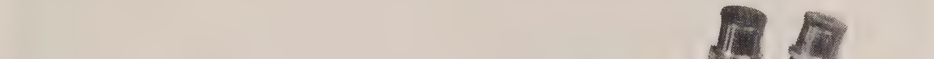

Fig. 2

G. R. F. HILSON—A SIMPLE MICROFORGE. PLATE 1

Gledhill, A. W. (1952). *J. gen. Microbiol.* 7, 179–191

The Immunizing Antigens of *Erysipelothrix rhusiopathiae*. The Role of the L-Antigen

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SUMMARY: Vaccines were prepared by formolizing cultures of *Erysipelothrix rhusiopathiae* and precipitating with aluminium hydroxide gel (AG). Fifty per cent of mice were protected against about a thousand lethal doses. Two (Ru and VYS) of eleven strains yielded highly potent vaccines.

Serum in the medium increased the potency of vaccines and for one of the strains liver extract was needed in the medium to give potent vaccines. In general, vaccines protected well against heterologous strains but protection was relatively poor against one of these.

A soluble antigen present in culture fluids induced in rabbits antibodies protecting mice and agglutinating suspensions of *E. rhusiopathiae* grown in the presence of serum. This soluble antigen was thermo-labile. In these respects it resembles the L-antigen, differing only in being detached from the surface of cells. Soluble antigen was precipitated with AG, yielding fairly good vaccines. Cells freed from media were also precipitated, yielding rather better vaccines. It is suggested that cells yield vaccines of high potency when they have produced and retained a relatively large amount of L-antigen.

Merthiolate-killed cells of *Erysipelothrix rhusiopathiae* grown in serum-containing media are capable of inducing in rabbits circulating antibodies protective for mice (Gledhill, 1947). Preliminary experiments with pigs indicated that whereas some circulating antibodies were also produced by intravenous injection of killed cells, a stronger response was elicited by suspensions of formalin-killed cultures from which most of the cells had been removed by centrifugation (Gledhill, 1948). Traub (1947) showed that mice and pigs could be actively immunized with cultures precipitated with aluminium hydroxide and that both cells and a soluble antigen present in cultures were necessary for producing fully active vaccines. Moreover, such active vaccines were only produced by a few strains of *E. rhusiopathiae.* However, Traub did not give the technical details of his methods. The purpose of this investigation is (1) to confirm that killed vaccines precipitated by aluminium hydroxide gel immunize mice effectively; (2) to confirm the existence of Traub's soluble antigen; (3) to determine its relationship to the L-antigen developed on the surface of cells grown in serum-containing media (Gledhill, 1947).

METHODS

Maintenance of strains. In general, strains were maintained on serum agar slopes and subcultured at monthly intervals. Good immunizing strains were lyophilized. Strains for challenging mice were passaged through normal mice at frequent intervals and cultures made on serum agar slopes were kept at

4° and used when not more than 3 days old for seeding cultures for challenging mice.

Media. Meat infusion broth prepared from horse flesh and Wright's (1933) infusion broth referred to as HW broth, were used. For ordinary vaccine production 2–2·5 % (v/v) horse serum was added to HW broth. For some vaccines, 10 % extract of fresh ox liver was also incorporated.

Preparation of aluminium hydroxide gel (AG). Solution 1: 300 g. ammonium sulphate were dissolved in 340 ml. hot distilled water, cooled to 60° and then filtered. Solution 2: 25 g. aluminium sulphate were dissolved in 100 ml. boiling distilled water and allowed to cool to 60°. To solution 1 was added 80 ml. conc. ammonia (sp.gr. 0·880) and then solution 2 was added immediately and stirred for 15 min. The precipitate was washed with distilled water, with 0·012 N-HCl and again with distilled water. The pH value was adjusted to 7·6 and the precipitate taken up in 60 ml. distilled water and autoclaved.

Preparation of vaccines. Horse serum HW broth or medium modified as stated in the text, was warmed to 37° and seeded with the appropriate strain. After 20–24 hr. incubation at 37°, when the opacity of the culture was about equal to that of a no. 2 Brown's tube (Burroughs Wellcome and Co., London) formalin to 0·4–0·5 % (v/v) was added and the whole allowed to stand in the laboratory for 24 hr. The culture was then tested for sterility and put at 4° for another 24 hr. For every 100 ml. medium, 4 ml. AG were added and well mixed in, followed by another 1 ml. AG a few hours later. After standing a further 18 hr. at 4°, the material was lightly centrifuged to pack the deposit and the clear supernatant withdrawn. In the earlier vaccines 95 ml. supernatant were withdrawn but in later vaccines 90 ml. were withdrawn to give about 10 ml. vaccine for every 100 ml. medium. The completed vaccine was tested for sterility.

Filtration of cultures. Liquid media were centrifuged at 3000 r.p.m. for ½ hr. and the supernatant filtered with positive pressure through a Gradocol membrane of average pore diameter (APD) 1·0 μ. standing on a finer membrane of APD 0·6 or 0·7 μ. as required. For filtration through finer membranes of APD 0·2 and 0·1 μ., the supernatant fluid was first filtered through membranes of APD 1·0 μ. and 0·6–0·7 μ., as described, and the filtrate was then passed through the finer membranes. In this way, 100 ml. volumes were obtained from one set of filters. To filter 5 ml. quantities it was possible to omit the centrifugation. Filtrates were usually treated with formalin as an added precaution of sterility.

Immunization of rabbits. Rabbits were immunized by four intravenous injections at 4-day intervals and bled 10 days after the last injection. For this purpose, filtrates were given in 1, 2, 3 and 3 ml. doses and cells, standardized to Brown's no. 3 tube and suspended in 0·5 % formol saline, in ½, 1, 1½ and 1½ ml.

Tests of vaccines in mice. Groups of mice (16–18 g.) were injected subcutaneously in the back, each mouse with a 0·2 ml. dose of fourfold dilutions of vaccine from 1/1 to 1/1024. Dilutions of vaccine were made with undiluted AG in early experiments and afterwards with AG diluted 1/2 with sterile water. Controls were set aside and, in many cases, injected with the same dose of diluted AG. It soon became clear that AG had no effect upon resistance to subsequent challenge. Fourteen days after vaccination, mice were challenged with the appropriate strain, usually the homologous one. The challenge strain was seeded from a serum agar culture (as already described) to 50 ml. 1 % (v/v) serum HW broth. After growing the culture for 18–21 hr., it was centrifuged and the organisms suspended in HW broth to an opacity equal to Brown's tube no. 8. Tenfold dilutions were then made in HW broth to give a final suspension of opacity $10^{-4} \times$ Brown's tube no. 8. This suspension was then injected intraperitoneally 0·5 ml./mouse, the cells present representing more than 10^3 M.L.D. Sickness and deaths of mice were recorded daily for 14 days, when the experiments were terminated. As few mice which became sick recovered, results are given in terms of mortalities only. The majority of mice which died did so within 1 week. For a satisfactory test the controls should die within 4–5 days. When this time of survival is greatly exceeded, the results tend to become erratic, sick mice often recovering without relation to the dilution of vaccine which they had received. In some cases the mortalities for each dilution of vaccine have been set out in tables but in the majority of cases the results have been expressed as the PD 50, that is the reciprocal of the dilution of vaccine giving 50 % survival of mice, calculated according to the method of Reed & Muench (1938).

Tests of rabbit sera in mice. Mice (18–20 g.) were injected with rabbit sera intraperitoneally. For tests of sera from rabbits immunized with culture filtrates, the dose given to each mouse was 0·3 ml., since the purpose was to detect protective antibodies in rabbits which had received minimal quantities of antigen. For tests of sera against suspensions of killed cells, the dose given was 0·1 ml. serum diluted with 0·2 ml. saline, to give a gross dose of 0·3 ml. as before. Twenty-four hr. later, mice were challenged by intraperitoneal inoculation of the dose of cells used for challenging vaccinated mice. The effects of challenge were recorded in the same way as for the challenge of vaccinated mice.

Agglutination tests. These were carried out according to the technique previously described by Gledhill (1945).

EXPERIMENTAL

The preparation of effective killed vaccines

Preliminary selection of strains. Vaccines were prepared against nine strains of *E. rhusiopathiae* from growth obtained by cultivation for 24 hr. in 50 ml. medium made up as follows: meat broth, 40 ml.; glucose, 0·5 g.; Hartley's broth, 5 ml.; sodium oleate, 3·0 mg.; serum ultrafiltrate, 5 ml.

This medium was used because it contained no serum and yet showed some capacity to grow bacterial cells which, after killing with formalin, were able to produce circulating protective antibodies when injected into rabbits. The growths, of opacity about equal to no. 1 Brown's tube, were treated with formalin (0·5 %, v/v), allowed to stand at room temperature for 24 hr., tested for sterility and precipitated at 4° with 2 ml. aluminium gel preparation (AG). After light centrifuging the supernatant was decanted to give 2·5 ml. vaccine. These vaccines were then diluted 1/4 and 1/16 with AG and injected subcutaneously into the backs of mice in 0·2 ml. doses. Fourteen days later, the mice, together with normal controls, were challenged with about 1000 lethal doses of the homologous strain. It will be seen from Table 1 that the vaccine made with strain Ru was sharply divided from the rest in its capacity to prevent the death of four out of five mice at dilution 1/4 and three out of six mice at dilution 1/16. Of the other vaccines, perhaps those from EW 16 and CS showed some slight activity in lengthening the survival time of vaccinated mice, as compared with controls; the remaining six gave no evidence of any activity.

Table 1. *The activity of vaccines from nine unselected strains*

	Vaccine dilutions				Control mice	
	1/4	1/16	1/4	1/16		
Strain designation	No. mice dying/group		Av. time to death (days)		No. mice dying/group	Av. time to death (days)
Ru 1/50	1/5	3/6	6·0	3·3	5/5	2·8
CS	6/6	6/6	6·5	6·1	6/6	3·8
EW16	3/4	5/5	6·3	4·0	6/6	4·6
R	5/5	6/6	5·4	4·0	6/6	4·0
EW24	4/4	6/6	2·8	3·3	6/6	2·8
MEW22	4/4	6/6	3·5	3·3	8/8	3·0
Chile	5/5	6/6	4·0	7·1	6/6	5·3
Rox	3/3	6/6	4·3	2·5	5/5	4·2
WW1	5/5	6/6	2·2	3·0	6/6	3·0

Mice were injected 0·2 ml. subcutaneously dilutions of vaccine made in AG and challenged 14 days later with the usual challenge dose of the homologous strain.

The influence on vaccines of serum in the medium. In a subsequent experiment using two vaccines (Ru 2/50 and 3/50) prepared from strain Ru grown in another batch of the above complex medium, growth was delayed, although after 48 hr. the opacity was equal to about no. 1 Brown's tube. Tests with both these vaccines showed neither to have any activity whatsoever, whereas the activity of the original vaccine Ru 1/50 was confirmed, none dying of five mice immunized with vaccine diluted 1/64. The delay in growth suggested that the failure of these two vaccines may have been due to the unsuitability of the medium. A vaccine (Ru 4/50) was therefore prepared using 2 % (v/v) serum HW broth and this was found to stimulate protective antibody even when diluted 1/256. The question was further tested by preparing another vaccine (Ru 5/50) in the original medium and comparing it with a vaccine (Ru 6/50) prepared at the same time in medium in which the 5 ml. serum ultrafiltrate were replaced by 2 ml. serum and 3 ml. water. The PD 50's were 8 for vaccine Ru 5/50 and 52 for vaccine Ru 6/50. For the production of subsequent vaccines, horse serum was incorporated into the medium and, using strain Ru, all proved consistently satisfactory. The number of these vaccines is twelve, representing productions of vaccine on five separate occasions during a period of 12 months. Furthermore, three satisfactory vaccines have been prepared independently by Mr A. Thomson, M.R.C.V.S., of the Wellcome Research Laboratories, Beckenham, with a view to immunizing pigs.

It was observed that the rate of growth of the mice given vaccine was less than the rate of normal mice and some of the vaccinated mice died. To meet this difficulty all vaccines after Ru 6/50 prepared by precipitation with AG were made to a final volume of 10 ml./100 ml. medium, instead of 5 ml./100 ml. medium. Also, in testing them with mice, dilutions were carried out with the AG diluted 1/2 with water.

Influence of liver extract in the medium for vaccine production. Using another strain of organism (VYS), obtained from Dr A. W. Stableforth and believed to be derived from that used for vaccine production in Yugoslavia, one vaccine was prepared using 2½ % (v/v) serum HWB medium (vaccine VYS 6/51) and another using medium prepared from 20 % ox liver and 80 % horse meat, instead of horse meat only (vaccine VYS 8/51). The PD 50's against the homologous strain were 7 for VYS 6/51 and 256 for VYS 8/51, showing the advantage of liver extract in preparing vaccine with strain VYS. On the other hand, with otherwise similar batches of media made with and without liver, and inoculated with strain Ru, the PD 50's were 47 for the vaccine from medium containing liver extract and more than 256 for vaccine from the medium not containing liver. This suggests that liver extract in the medium had no advantage in the case of strain Ru. Another vaccine prepared with strain VYS using 2½ % (v/v) serum HW broth to which was added 10 % beef liver extract had a PD 50 of 26, confirming that the strain was capable of producing reasonably efficient vaccines.

The results of the experiments described show that the production of effective vaccines greatly depends upon the constitution of the medium used and that there may be differences between the growth requirements of different strains. Most strains appear incapable of giving effective vaccines, thus confirming the work of Traub (1947). Nevertheless, it might well be that of the nine strains originally tested, more than one would have yielded an efficient vaccine if the medium had contained serum and liver extract. A vaccine prepared with strain EW 24 grown in 2½ % (v/v) serum HW broth had a PD 50 of 4, whereas vaccine prepared with this strain grown in the original medium containing no serum gave no protection to mice at a dilution of 1/4 (see Table 1).

It has been stated that some strains agglutinate fowl red blood cells and that among these strains are to be found those which can produce vaccines (Dinter, 1948; Bakos, 1951). In a limited number of tests, it was found that agglutination depended not only upon the strain of organism used but also upon the particular fowl giving the red blood cells, and upon the medium used for growth. The few tests carried out did not seem to confirm the value of haemagglutination for the selection of good vaccine-producing strains.

The effect of filtration. On occasions cells of *E. rhusiopathiae* have passed through bacterial filters during the preparation of biological products and this has led to disastrous results (Edwards, 1949; Rowlands & Edwards, 1950). The filtrability of the strains of the organism was tested primarily to determine whether the antigen present in supernatants after centrifuging and treating with formalin (Gledhill, 1948) and after filtration (Traub, 1947) might really be small filter-passing cells (Gledhill, 1950).

Cultures in HW broth were filtered through Gradocol membranes of APD 0·6–1·0 μ. and 5 ml. filtrate were used to inoculate 30 ml. of the same medium. Of eleven strains tested in this way, growths appeared in seven filtrates through membranes of 0·8 μ. APD or less. Re-tests, when performed, gave consistent results except in the case of one strain (Chile); details of these filtrations are shown in Table 2. The question as to whether filter-passing cells are solely responsible for the antigenic activity of filtrates is considered later. Here, it is noted that a vaccine prepared from strain CS had a PD50 of 20 when growth was initiated from filtrate, whereas inoculation of unfiltered organisms into the batch of medium yielded a vaccine with a PD50 of 4. It is also of interest that strain EW2 with which the antigenic activity of supernatant after centrifugation and sterilization was first observed (Gledhill, 1948) and which subsequently lost this activity, was not then filtrable through a membrane of APD 0·8 μ. or less. However, growth from an ampoule of this strain dried in 1942 was found to be filtrable. Vaccines prepared from growths from filtrates and from ordinary seeding of lyophilized culture both had a PD50 of 25. There are therefore indications that filtrability may have some bearing upon vaccine production. The explanation might be that a majority of cells fully in the S-phase are required for vaccine production and that such cells are more filtrable than R-forms. If this be so, occasional filtration of vaccine-producing strains, and seeding of medium with such filtrates might tend to prevent loss of power to yield active vaccines.

Table 2. *The filtrability of strains of* E. rhusiopathiae

Strain designation	APD of Gradocol membrane (μ.)	No. of filtrates not sterile/no. filtrations performed
Ru	0·76, 0·8	2/2, 2/2
VYS	0·68	3/3
EW2	0·8	0/2
MEW22	0·8	1/1
EW16	0·8	0/2
EW24	0·8	1/1
R	0·8	0/1
Rox	0·8	0/1
CS	0·8	2/2
Chile	0·8	1/3
WW1	0·8	1/1

Cross immunity of vaccinated mice. Vaccines Ru 1/51 and VYS 8/51 were injected as usual into groups of mice which were challenged fourteen days later with the usual dose of strains Ru, VYS, EW2, V and CYS. Strains EW2 and V were selected because they are virulent representatives of groups II and III respectively, whereas Ru belongs to group I, the classification being based upon quantitative somatic antigens (Gledhill, 1945). The group of VYS and CYS has not been determined; CYS was selected as being the strain used in Yugoslavia for testing in mice vaccines prepared with strain VYS. The results are shown in Table 3. It will be seen that with the exception of vaccinated mice challenged with CYS, both vaccines protected against heterologous strains, even when highly diluted. The tests were carried out over a period of 2½ months during which time the vaccines were stored at 4°.

The existence and relationship to L-antigen of soluble antigen present in culture fluids

The existence of soluble antigen. The existence of a soluble antigen was suspected when it was found that formolized supernantants obtained by centrifuging cultures of strain EW 2 were not only capable of stimulating rabbits to produce protective antibodies but also pigs, in which animals the bacterial cells themselves produced few antibodies (Gledhill, 1948). Traub (1947), however, claimed to have established the existence of a soluble antigen capable of adsorption and precipitation by AG. Vaccines so produced immunized mice although not usually at so high a dilution as vaccines prepared by the precipitation of cells. The best vaccines resulted from the precipitation of both soluble substance and cells. As already remarked, the filtrability of *E. rhusiopathiae* suggested the possibility that the antigenic activity of sterilized supernatants might be due to the small undeposited cells in them. This possibility seemed to receive confirmation from the high antigenic activity of small cells obtained by high-speed centrifugation (20,000 r.p.m. for 1 hr.) of supernatants obtained after ordinary centrifugation. This activity was found, however, to be no greater than that of an approximately equal number of cells deposited by slow centrifugation. Furthermore, if sterilized supernatants were antigenic in virtue of the small cells in them, filtrates might be expected to be antigenic in virtue of the *viable* small cells in them, even though very few in number (Gledhill, 1950). In earlier tests with strain EW 2, formolized filtrates did not have any antigenic activity, but later tests with strains Ru and VYS showed them to possess sufficient antigenic activity to produce, at the doses used, a measurable amount of circulating antibody in rabbits (Tables 4 and 6). In the following section it is further shown that sterile filtrates treated with AG yielded a precipitate capable of immunizing mice, thus confirming Traub's claim.

The relationship of soluble antigen to L-antigen. Thermolabile L-antigen produced on the surface of cells grown in media containing serum and inactivated with merthiolate, evokes in rabbits circulating antibody capable of protecting mice and agglutinating suspensions of cells grown in the same media, after adsorption with cells grown in media devoid of serum (Gledhill, 1947). Throughout that work the basal medium, to which serum was added, was ordinary meat broth, prepared from fresh horse meat or from a commercial dried preparation with 1 % Evans peptone. Glucose was also added to a concentration of 1 % in the media devoid of serum. While it has been confirmed that such a basal medium is rarely capable of growing cells which, after being killed by formalin or merthiolate, evoke in rabbits circulating antibodies

Table 3. *The immunizing power of vaccines against homologous and heterologous strains*

Designation of challenging strain	Vaccine dilutions (Ru 1/51) 1/1	1/4	1/16	1/64	1/256	1/1024	Approximate PD 50 of vaccine	Vaccine dilutions (VYS 8/51) 1/1	1/4	1/16	1/64	1/256	1/1024	Approximate PD 50 of vaccine	Controls, no. mice dying/group
	No. mice dying/group							No. mice dying/group							
Ru	1/5	1/5	0/5	1/5	1/5	4/5	512	0/5	0/5	1/5	1/5	3/5	5/5	135	10/10
VYS	0/5	0/5	0/5	1/5	2/5	2/5	> 1024	0/5	0/5	0/4	0/5	3/5	4/5	256	9/9
EW2	1/5	0/5	2/5	2/5	4/5	nt	100	0/5	nt	0/5	2/5	2/5	nt	> 256	9/10
V	1/5	0/5	1/5	0/5	3/5	nt	203	1/5	1/5	1/5	0/4	4/5	nt	152	9/9
CYS	1/5	0/5	4/5	4/5	3/4	nt	10	2/4	0/5	3/4	5/5	4/5	nt	7	9/9

Mice were injected 0·2 ml. subcutaneously dilutions of vaccine made in AG and challenged 14 days later with the usual challenge dose of the homologous or heterologous strains. nt = not tested.

Table 4. *Protective sera produced in twelve rabbits by formolized filtrates of strain Ru*

APD of membrane (μ.)	No. of passively immunized mice dying/group	Controls, no. mice dying/group
1·0	1/6	11/11
1·0	2/6	11/11
0·68	2/6	11/11
0·68	2/6	11/11
0·66	0/5	10/10
0·66	2/5	10/10
0·66	3/5	10/10
0·23	4/6	11/11
0·23	0/6	11/11
0·11	1/5	10/10
0·11	0/5	10/10
0·11	1/5	10/10

sufficient to protect mice, nevertheless Hartley's tryptic digest broth, especially when containing 1 % glucose, and Wright's broth, are capable of growing cells antigenically active after being killed (Table 5). Although satisfactory growths for vaccine production can sometimes be obtained in media without serum, the presence of serum is advantageous. This similarity in respect of serum in the medium for vaccine production and for producing L-antigen suggested that L-antigen upon the surface of cells might also be free in the medium and that its presence in one or the other *locus* was essential for producing active vaccine. Support for this hypothesis has been obtained in the following three ways.

Table 5. *The protective power for mice of sera prepared by immunizing rabbits with killed suspensions of strain EW 2 grown in meat broth, Hartley's broth and Hedley Wright's broth*

Medium used for growing immunizing cells	1 % glucose added to medium	No. of passively immunized mice dying/group		Controls, no. mice dying/group
Meat broth	−	6/6		6/6
	+	6/6	23/24	12/12
	+	5/6		
	+	6/6		
	+	6/6		
Hartley's broth		4/6	15/24	18/18
	−	5/6		
	−	2/6		
	−	4/6		
	+	0/6	7/24	12/12
	+	4/6		
	+	0/6		
	+	3/6		
Hedley Wright's broth	−	0/6	4/23	11/11
	−	0/6		
	−	1/6		
	−	3/5		

Mice were passively immunized with 0·1 ml. intraperitoneally of rabbit serum and were challenged 24 hr. later with EW 2 in the usual challenging dose.

(*a*) While active vaccines can be produced from culture filtrates and while such filtrates evoke in rabbits circulating antibodies able to protect mice, such filtrates after heating fail to manifest activity in either respect. Volumes (200 ml.) of serum (2·5 %, v/v) HW broth containing 10 % (v/v) beef liver extract were seeded with strain Ru or strain VYS and incubated for 20 hr. at 37°. The cultures were then centrifuged and filtered through Gradocol membranes of 1·0μ. APD on membranes of 0·61μ. APD. The filtrates from each strain were divided into two portions of 100 ml. and one portion heated to 64° for 1 hr. Although it was known that a temperature of 75° was necessary to destroy L-antigen completely, it was impossible to use a temperature higher than 64° owing to the serum present in the filtrates. Formalin was then added to heated and unheated filtrates to give a concentration of 0·4 % (v/v). To 50 ml. of each portion, 2·5 ml. AG was added and 5 ml. vaccine prepared. The remainder of each portion was used to immunize three rabbits by intravenous injection at 4-day intervals with 1, 2, 3 and 3 ml. doses, the rabbits being bled for serum 10 days after the last injection. The results of testing the power of the vaccines to immunize mice actively and of the sera to immunize them passively are shown in Table 6. They clearly support the hypothesis that L-antigen is the active principle in filtrates.

(*b*) Sera from the above rabbits were also found to be capable of agglutinating suspensions of cells grown in serum HW broth at titres from 1/20 to 1/160, a fact which shows the filtrates to contain an antigen closely similar to that of the cells. Furthermore, the serum of highest titre against filtrate of each strain was tested for its power to agglutinate cells of strains Ru and VYS grown in ordinary broth media with and without serum. From Table 7 it will be seen that the suspensions grown in medium containing serum were agglutinated, whereas there was no agglutination at 1/20, the lowest dilution, in suspensions grown in medium not containing serum. This result also conforms to the hypothesis that filtrates contain L-antigen.

(*c*) Cells freed from medium can produce active vaccines, and circulating protective antibodies in rabbits. After heat treatment such cells fail in both respects. That cells can produce active vaccine after centrifugation and re-suspension in saline or fresh medium has been shown seven times with strain Ru, the PD 50's being 19, 35, 6, 23, 4, 37 and 64. In one case (PD 50 = 23) the cells had been washed three times before being finally suspended in saline. Three vaccines were prepared with VYS cells and these gave PD 50's of 4, 81 and 203. Heating saline suspensions of strains Ru and VYS grown in serum HW broth to 64° for 1 hr. rendered them incapable of evoking in rabbits protective antibody for mice, whereas unheated portions of the same suspensions after inactivation with formalin did evoke antibody. Thus, the mortality was six out of six mice challenged after immunization with sera from each of the three rabbits immunized with heated Ru cells (18/18) as against nil, one, and two out of six mice with sera from each of the corresponding rabbits immunized with unheated Ru cells (3/18). For strain VYS, the mortalities of mice challenged after receiving sera against heated cells were five, six and six out of six mice (17/18) as against nil, nil and one out of six mice (1/18). The vaccines prepared with both the Ru and VYS cells, whether heated or not heated, protected 50 % of mice when diluted 1/4. This result would suggest the possibility that a thermostable antigen rather than L-antigen was responsible for the activity of vaccines prepared with cells. However, in view of the fact that the heating was insufficient to destroy the L-antigen it was decided to make fresh vaccines against heated and unheated cells of strains Ru and VYS, the temperature of heating being raised to 80° for ½ hr. The PD 50's of the heated vaccines were <1 for strain Ru and for strain VYS as against PD 50 values of 37 for strain Ru and 81 for strain VYS of the corresponding unheated vaccines. In another similar experiment the PD 50's were 1 for strain Ru and 6 for strain VYS as heated vaccines against 64 and 203 for the corresponding unheated vaccines. Having regard to these results and to the low grade activity of the vaccines from the unheated cells in the former

Table 6. *Heated and unheated filtrates for immunizing mice and rabbits*

			Vaccinated mice					Mice protected with immune rabbit sera	
			Vaccine dilutions						
			1/1	1/4	1/16	1/64			
Immunizing substance	Heat treatment (64° for 1 hr.)	Designation of vaccine	No. of mice dying/group				Approximate PD 50	Rabbit nos.	No. of mice dying/group
Ru filtrate (0·61 μ.)	–	Ru 13/51	0/4	2/5	4/5	2/5	11	37, 38, 39	2/6, 1/6, 1/5
Ru filtrate (0·61 μ.)	+	Ru 16/51	2/5	4/5	5/5	nt	2	43, 44, 45	6/6, 6/6, 6/6
VYS filtrate (0·61 μ.)	–	VYS 19/51	1/5	1/5	3/5	4/5	10	49, 50, 51	1/6, 3/6, 2/6
VYS filtrate (0·61 μ.)	+	VYS 22/51	4/5	5/5	5/5	nt	<1	55, 56, 57	5/6, 5/6, 6/6

Vaccinated mice were challenged 14 days after vaccination and passively immunized mice 24 hr. after receiving 0·3 ml. rabbit serum intraperitoneally, the usual dose of challenge organisms being used. Forty-one controls of the challenge cultures died in average times of 2·6 and 3·9 days for Ru and VYS respectively.

Table 7. *The agglutination of Ru and VYS grown in media with and without serum by sera from rabbits immunized with filtrates of their growths*

		Agglutinating sera of rabbit immunized with filtrate							
		Ru				VYS			
Strain designation	Serum present in growth medium of agglutinated cells	1/20	1/40	1/80	1/160	1/20	1/40	1/80	1/160
Ru	Present	++++	++++	++++	++++	++++	+++	·	·
Ru	Absent	·	·	·	·	·	·	·	·
VYS	Present	++++	++++	+++	++	++++	++++	++	·
VYS	Absent	·	·	·	·	·	·	·	·

Serum-antigen mixtures incubated at 42° for 4 hr. and final reading taken after a further 20 hr. incubation at 37°. + = trace agglutination; ++ = agglutination with some settlement; +++ = agglutination with almost total settlement; ++++ = agglutination and total settlement.

experiment, it seems reasonable to suppose that L-antigen plays an important rôle in vaccines of high activity prepared with cells. Indeed, it may be that the amount of L-antigen retained by cells is somewhat variable and also the thermostable somatic antigen in itself may yield a vaccine of low activity. If these surmises were correct, then unheated cells giving a vaccine of low activity would probably be cells which had retained little L-antigen and would give a vaccine of almost equal activity after being heated; whereas cells giving a vaccine of high activity would be cells which had retained much L-antigen and so give a vaccine of much reduced activity after heat treatment.

DISCUSSION

The experiments described confirm Traub's claims that inactivated vaccines capable of effectively immunizing mice can be prepared from cultures of *E. rhusiopathiae*; that only a proportion of strains yield active vaccines; and that soluble antigen, present in the culture fluid after growth, is a necessary component of active vaccines. Reasons have been advanced to show that this soluble antigen is probably the thermolabile L-antigen previously described in association with cells. In cultures capable of yielding highly active vaccines, it seems likely that the ability of the cells to give active vaccines after separation from the culture fluid depends upon the extent to which they have retained L-antigen upon their surfaces. Conversely, filtrates give active vaccines in proportion to the extent that the cells present before filtration had released L-antigen into the medium.

The reason why only a minority of strains yield active vaccines is not clear. Presumably strains which yield active vaccines are strains which produce a sufficiency of fully developed L-antigen in the medium used, whereas strains which do not yield active vaccines do not produce sufficient fully developed L-antigen. Strains not believed to be capable of yielding active vaccines do, however, produce L-antigen when grown in serum-containing media which is sufficient in quantity or quality to render them agglutinable by anti-L sera (Gledhill, 1947). The fact that although on one occasion strain Ru produced active vaccine in a medium devoid of serum and on other occasions failed to do so, and the fact that strain VYS seems to require liver extract, suggest that all strains in the S-phase might yield active vaccines provided the medium used were suitable. The capacity of HW broth to grow cells of strain EW2 which, after killing, produce protective antibodies in rabbits, contrasts with the capacity of ordinary broth, which does not yield cells having this property. The essential difference between HW broth and ordinary broth appears to be that the peptone is added to the former before the meat juices are expressed and boiled, whereas in ordinary broth peptone is added to the complete meat extract. Investigation of the possible toxicity of peptone in relation to the development of L-antigen might lead to a medium capable of growing most strains to yield it satisfactorily.

I wish to thank Dr A. W. Stableforth for providing me with two valuable strains of *E. rhusiopathiae*, Mr A. Thomson, M.R.C.V.S., for allowing me to refer to tests of his vaccines in mice, and the late Dr W. J. Elford for providing serum ultrafiltrate. I also gratefully acknowledge the technical assistance of Mr A. Coe.

REFERENCES

Bakos, K. von (1951). Über Herstellung und Prüfung eines konzentrierten Adsorbatimpfstoffes gegen den Schweinrotlauf. *Nord. vet. Med.* **3**, 109.

Dinter, Z. von (1948). Über den Haemagglutinationshemmungstest beim Rotlauf. *Berl. Münch. tierärztl. Wschr.* **10**, 113.

Edwards, C. M. (1949). Polyarthritis in lambs following serum inoculation. *Vet. Rec.* **61**, 95.

Gledhill, A. W. (1945). The antigenic structure of Erysipelothrix. *J. Path. Bact.* **57**, 179.

Gledhill, A. W. (1947). Some properties of a thermolabile antigen of *Erysipelothrix rhusiopathiae*. *J. gen. Microbiol.* **1**, 211.

Gledhill, A. W. (1948). Reprinted from discussion: swine erysipelas infection (*Erysipelothrix rhusiopathiae*) in man and animals. *Proc. R. Soc. Med.* **41**, 330.

Gledhill, A. W. (1950). Immunization against *Erysipelothrix rhusiopathiae* with killed vaccine. *Nature, Lond.* **166**, 952.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty per cent end points. *Amer. J. Hyg.* **27**, 493.

Rowlands, W. T. & Edwards, C. M. (1950). Investigation of further cases of polyarthritis in lambs following serum inoculation. *Vet. Rec.* **62**, 213.

Traub, E. (1947). Immunisierung gegen Schweinerotlauf mit konzentrierten Adsorbatimpfstoffen. *Mh. Veterinarmed.* **2**, 165.

Wright, H. D. (1933). The importance of adequate reduction of peptone in the preparation of media for the pneumococcus and other organisms. *J. Path. Bact.* **37**, 257.

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Sharpe, M. Elisabeth (1952). *J. gen. Microbiol.* 7, 192–199

Occurrence of a Common Type Antigen in Streptococci of Groups D and N

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SUMMARY: A streptococcus reacting with both group D and group N specific antisera was found to belong to group D; the reaction with the group N serum was shown by reciprocal absorption tests to be due to a common type antigen. Two group D strains were encountered having this antigen; both were identified as *Streptococcus faecium*. The group N strain possessing this same antigen was *Streptococcus lactis*. It proved to have at least one other type antigen, also found in another strain of *Strep. lactis* which was not serologically identical. Two antigenic variants were present in cultures of this organism; one possessed all the type antigens characterizing this strain the other had only one. Attempts to cause further dissociation or reversion were not successful.

Streptococci of different Lancefield groups having common type antigens have been previously reported; Bliss (1937) found a group F type to be serologically identical with a group G type, and Maxted (1949) described four strains of streptococci, one belonging to group B, two to group C and one to group G, each having the M antigen of group A, type 28. When a streptococcus was encountered which reacted with both a group D and a group N specific antiserum in the precipitin test, using formamide extracts, it seemed likely that one of the reactions might have been due to a common type antigen. This was found to be so, and the following account is given of a group D and a group N streptococcus having a similar type antigen. Later another group D strain was found with this same antigen.

MATERIALS AND METHODS

The streptococcus strain 748, an extract of which reacted with a group D and a group N serum, was sent to this laboratory by Prof. M. Seelemann, Institut für Milchhygiene, Kiel. It was isolated from the blood of a case of endocarditis. Prof. Seelemann was also kind enough to send samples of his group D and group N sera, and the strains group D 753 (Seelemann & Carstens, 1951*a*) and group N 736 (Seelemann & Carstens, 1951*b*) from which they had been prepared.

Group D streptococcus 2766/1 was isolated from a Gruyère cheese from Cyprus by Dr B. C. Hobbs, Central Public Health Laboratory, Colindale, and sent to this laboratory for investigation.

Cultural tests

Growth at 45°; survival at 60° for 30 min. (Shattock, 1949).
Growth at pH 9·6 (Shattock & Hirsch, 1947).
Growth at 40°; hydrolysis of arginine (Abd-el-Malek & Gibson, 1948).
Resistance to 1:2500 *potassium tellurite* (Sharpe & Shattock, 1952).

Carbohydrate fermentation. Peptone water containing 0·5 % (w/v) of the carbohydrate added after sterilization.

Strains 748 and 2766/1 were incubated at 37° and strain 736 at 30° for all tests unless otherwise stated.

Serological methods

Preparation of antisera. Type sera. Formol-killed suspensions were inoculated into rabbits at twice weekly intervals for 3–4 weeks (Sharpe & Shattock, 1952).

Group sera. Group D. The organisms were disrupted in a vibrating machine with carborundum (Shattock, 1949), and a course of four to six inoculations given at twice-weekly intervals. Group N. An 18 hr. culture grown in glucose (1 %) Lemco broth was centrifuged, washed in 0·85 % (w/v) saline and resuspended to its original volume in saline; it was then heated to 56° for 1 hr. Twelve to fifteen inoculations were given at twice-weekly intervals using a freshly heat-killed suspension for each inoculation.

HCl *extracts.* Lancefield's (1933) method was used for preparing HCl extracts.

Formamide extracts. Fuller's (1938) method was used.

Precipitin ring tests. Extracts were layered over sera in tubes of 3 mm. internal diameter and examined at intervals up to 30 min.

Absorption tests. Using Shattock's (1949) technique, organisms were grown for 48 hr. in glucose Lemco broth and, after washing once in saline, the cells were resuspended in saline to give a density equivalent to 10 times Brown's opacity tube no. 10 (Burroughs Wellcome and Co. Ltd.). Suspensions of group D streptococci were killed by heating at 60° for 1 hr. and the group N streptococci by heating at 56° for 1 hr. The cells were then packed by centrifuging and suspended in undiluted serum so that the absorbing suspension had 50 times the opacity of Brown's tube no. 10. Sera were absorbed for 1 hr. at 37° followed by overnight refrigeration. To ensure complete removal of antibodies, type sera were always reabsorbed with fresh organisms.

RESULTS

Serological identification of strain 748

HCl extracts of strain 748 gave precipitin reactions with two specific group D sera, *Strep. durans* 98 D and *Strep. bovis* Pearl 11, but not with a specific group N serum *Strep. lactis* OJ. An antiserum was prepared against strain 748, using the method for preparing group D sera, and found to react specifically with HCl extracts of strains of known group D streptococci, all of different serological types, and also with an extract of strain 736. Group sera prepared against strains 753 and 736 reacted specifically with extracts of group D and group N streptococci respectively. In addition serum 736 reacted with an extract of strain 748 (Table 1). Using Seelemann's sera similar results were obtained. Reciprocal absorption tests with serum 748 and a group D serum prepared against *Strep. durans* 98D showed that 748 cells absorbed all the group antibodies from the group D 98D antiserum, and that the 748 antiserum contained group antibodies which were absorbed by the group D strain 98D, but were not absorbed by the group N strain *Strep. lactis* OJ (Table 2). The streptococcus 748 is thus identified unequivocally as group D. This suggests that the precipitin reaction between 736 serum and an extract of strain 748 is due to a common type substance. The reaction between group D serum 753 and strain 748 is the normal group reaction.

Formamide extracts. Formamide extracts gave similar results to those recorded above, but the reactions were weaker. No further tests were made with extracts prepared in this way.

Table 1. *Precipitin reactions of antisera prepared against streptococci strains* 753, 748 *and* 736

	Antiserum		
HCl extract prepared from strain	753	748	736
Groups A–C, E–M	–	–	–
Group D:			
Strep. faecalis S161	+	+	–
N83	+	+	–
Strep. faecalis var. *liquefaciens* N97	+	+	—
Strep. faecalis var. *zymogenes* N37	+	+	–
Strep. bovis Pearl 11	+	+	–
Strep. durans 98D	+	+	–
Group N:			
Strep. lactis OJ	–	–	+
M3	–	–	++
Rice	–	–	+
Strep. cremoris HP	–	–	+
RW	–	–	+
J	–	–	+
Strain 753	+	+	–
748	+	+++	++
736	–	+++	+++

+Precipitin ring formed in 5–10 min.
++Heavy precipitin ring formed in 1–5 min.
+++Very heavy precipitin ring formed in 1 min.

Absorption of type specific antibodies from type antisera 748 *and* 736

Using type sera from which all non-type specific antibodies had been absorbed by a heterologous strain of the same group, reciprocal absorption tests were carried out with sera prepared against strains 748 and 736 (Table 3). With serum 748 the precipitating type antibodies were completely absorbed by either 748 or 736 cells, leaving no reacting antibodies in the serum. With 736 serum, although the precipitating antibodies were completely removed by absorption with the homologous strain, absorption with 748 cells still left some reacting antibody, although the precipitin reaction was greatly reduced. It had been observed (Table 1) that the group N serum prepared against 736 gave a strong reaction with an extract of *Strep. lactis* M3, and it was thought that this might be due to another type antigen present, differing from that characterizing 748 as an extract of *Strep. lactis* M3 did not react with 748 type serum. Accordingly, absorption tests with this organism were also included (Table 3), and the presence of a second type antigen was confirmed; the group N streptococcus

Table 2. *Reciprocal absorptions of group D serum 98D and serum 748, as shown by precipitin reactions*

HCl extracts prepared from group D strain	Unabsorbed	Absorbed with 98D (group D)	748	736	*Lactis* OJ (group N)
Absorption of group D serum 98D					
Strep. faecalis, 2 strains: S161 and N83	+	–	–	+	+
Strep. faecalis var. *liquefaciens* N97	+	–	–	+	+
Strep. faecalis var. *zymogenes* N37	+	–	–	+	+
Strep. bovis Pearl 11	+	–	–	+	+
Strep. durans 98D	+	–	+	+	+
Strain 753	+	–	–	+	+
748	+	–	–	+	+
736	–	nt	nt	nt	nt
Absorption of serum 748					
Strep. faecalis, 2 strains: S161 and N83	+	–	–	+	+
Strep. faecalis var. *liquefaciens* N97	+	–	–	+	+
Strep. faecalis var. *zymogenes* N37	+	–	–	+	+
Strep. bovis Pearl 11	+	–	–	+	+
Strep. durans 98D	+	–	–	+	+
Strain 753	+	–	–	+	+
748	+++	+++	–	+	+++
736	+++	+++	–	–	+++

nt = not tested.

Table 3. *Precipitin reactions showing the reciprocal absorptions of type specific antibodies from type sera prepared from group D strain 748 and group N strain 736*

HCl extract prepared from strain	Type specific serum 748: Un-absorbed	Type specific serum 748: Absorbed with 748	736	M3	Type specific serum 736: Un-absorbed	Type specific serum 736: Absorbed with 748	736	M3	M3 and 748
748, group D	+++	–	–	+++	++	–	–	++	–
736, group N	+++	–	–	+++	+++	++	–	+++	+
Strep. lactis M3, group N	–	–	–	–	++	++	–	–	–

736 was found to possess a type antigen similar to that of group N *Strep. lactis* M3 in addition to the 748 antigen. Absorption with M3, together with 748 cells, still failed to remove all antibodies in the 736 type serum, and it appeared that further residual antibodies were present which could not be identified. Exhaustive absorptions with both M3 and 748 cells, using 6, 24 or 48 hr. cultures for absorption still failed to remove the antibodies from the serum.

The major antigen appeared to be that shared by 748. Thus strain 736 contained one type antigen similar to that characterizing the group D streptococcus 748, another similar to that of the group N streptococcus *Strep. lactis* M3, and a third unidentified type antigen.

HCl extracts of several other group D strains were also tested against these two type sera and one of them, 2766/1, gave strong precipitin reactions. One-sided absorption of both 748 and 736 type specific sera showed that this streptococcus possessed the 748 antigen, but not the M3 antigen. HCl extracts of the serological types described by Sharpe & Shattock (1952) gave no precipitin reactions with type sera 748 or 736.

Physiological characters of strains of streptococci having the same type antigen

The physiological reactions of the three strains of streptococci sharing the 748 antigen are given in Table 4. The two group D strains are classified as *Strep. faecalis* or on the basis of their sensitivity to potassium tellurite and fermentation of arabinose as, *Strep. faecium* (Skadhauge, 1950; Seelemann & Carstens, 1951*a*; Sharpe & Shattock, 1952). Strain 736, previously described by Seelemann as *Strep. cremoris* would seem by the fermentation of maltose, the hydrolysis of arginine and ability to grow at 40° to be more closely allied to *Strep. lactis*, as Briggs & Newland (1952) also point out. The group N *Strep. lactis* M3 has already been fully described in the literature (Shattock & Mattick, 1943; Swartling, 1951).

Table 4. *Physiological characters of strains of group D and group N streptococci having the same type antigen*

	Strain		
	Group D		Group N
	S748	2766/1	736
Litmus milk (24 hr.)	rAC	AC	RA
Mannitol	A	A	A
Sucrose	—	—	—
Raffinose	—	—	—
Arabinose	A	A	—
Sorbitol	—	—	—
Maltose	A	A	A
Dextrin	—	nt	A
Trehalose	A	A	A
Growth at 40°	nt	nt	+
Growth at 45°	+	+	—
Growth at pH 9·6	+	+	—
Survival at 60° for 30 min.	+	+	—
Arginine hydrolysis	+	+	+
Haemolysis	Greening	α	Greening
Resistance to tellurite	—	—	—
Gelatin liquefaction	—	—	—

R = complete reduction; r = partial reduction; A = acid; C = clot; nt = not tested.

Antigenic variations of group N streptococcus strain 736

To investigate further the antigenic structure of strain 736, an 18 hr. glucose Lemco broth culture was plated on yeast dextrose agar and sixteen colonies picked off. HCl extracts of the growths from these were tested against type serum 736 unabsorbed, absorbed with *Strep. lactis* M3, or absorbed with strain 748. It was found that although there was no colonial differentiation, two variants of different antigenic make-up were present: one, 'A', having all type antigens previously described for strain 736, and the other, 'B', having only the *Strep. lactis* M3 type antigen. Absorptions of serum 736 were carried out with these two variants, and the results of several such experiments summarized in Table 5. Repeated platings of 736 cultures always showed the presence of the two antigenic forms and in all cases the same antigenic patterns were observed. Absorption of type serum 736 with the undifferentiated homologous organism removed all antibodies in the serum, and so did absorption with 736 'A'. Absorption with 736 'B' or with *Strep. lactis* M3 cells removed both the 736 'B' and *Strep. lactis* M3 antibodies. Further absorption of the serum previously absorbed with *Strep. lactis* M3 or 736 'B' cells removed the 748 type antibodies still leaving antibodies which reacted with extracts of 736 and 736 'A'. As 736 'A' cells removed all type antibodies they must have possessed all the 736 type antigens. Extracts of 736 'B' reacted exactly like *Strep. lactis* M3 in absorption tests and possessed only this type antigen. 736 has undergone variation, with the formation of a variant containing only one of the type antigens. All strains of 736 'A' and 'B' reacted with specific group N serum.

Table 5. *Antigenic relationships of serological variants of group N strain 736 shown by cross absorption tests*

		Type specific serum 736 absorbed with								
HCl extract of strain	Un-absorbed	736	736A	736B	736B and 748	M3	M3 and 748	748	Type specific serum 748 un-absorbed	Group N serum lactis OJ
736, group N	+++	−	−	+++	+	+++	+	++	++	+
736 A (15 strains)	+++	−	−	+++	+	+++	+	++	++	+
736 B (27 strains)	++	−	−	−	−	−	−	+	−	+
M3, group N	++	−	−	−	−	−	−	++	−	+
748, group D	++	−	−	++	−	++	−	−	++	−

Although two variants of strain 736 could be differentiated serologically, culturally and morphologically the differences were few. Colony forms on yeast glucose agar and on horse blood agar were indistinguishable even after ageing at room temperature or after prolonged incubation at 30°, being smooth in each case; the microscopic appearance was the same (short chains of Gram-positive cocci) except that the 'B' form tended to clump more than the 'A'. No capsules could be demonstrated in either variant. Fermentation of maltose and dextrin, growth at 40° and reactions in litmus milk were the same. The two forms were usually differentiated by their growth in glucose

Lemco broth, 'A' appearing as a diffuse smooth growth throughout the medium and 'B' as a rough granular deposit. On suspending washed cells in 0·85 % saline, 'A' cultures usually remained smoothly in suspension whilst 'B' cultures spontaneously agglutinated. However, a few of the 'A' cultures were also spontaneously agglutinated.

On subculturing four cultures of each variant in glucose Lemco broth daily for 10 days, the appearance in broth, serological reactions and physiological reactions remained unchanged. On replating an 'A' and a 'B' strain and picking eight colonies of each, the cultural and serological characteristics of all colonies of each variant were those of the parent strain. There was no further dissociation or reversion. Freeze-drying of an 'A' and 'B' strain also produced no change in antigenic or physiological properties. Both variants appeared to be stable under the conditions described, and no further attempts were made to determine conditions which might cause dissociation.

DISCUSSION

Strains 748 and 736 each have a type antigen stimulating the production of antibodies which react with extracts of both organisms. Strain 736 is able to remove all type antibodies from 748 antiserum by absorption, and strain 748 removes a large amount of the antibodies from antiserum 736. These type antigens are then very similar if not identical. In view of the antibodies remaining in 736 serum after absorption with *Strep. lactis* M3 and with 748 cells, it may be that strain 736 possesses a more complex antigen than strain 748 and that, whilst the 736 antigen can completely absorb the antibodies from 748 type serum, the 748 antigen can only partially absorb the similar but more complex antibodies from 736 type serum. Alternatively, the residual antibody may represent still another type antigen.

It is not known whether the type antigens of strains 748 and 736 are carbohydrate or protein; they are not inactivated by pepsin or trypsin.

Other group N streptococci may have the same intricate antigenic pattern; only by chance was strain 736 studied in such detail as to reveal its complexity. The serological reactions of the group D strain 748 are simple by comparison, revealing a single type and a group antigen. No tests were made for non-specific P substance.

Dawson, Hobby & Olmstead (1938) have described antigenic variation in haemolytic streptococci. These authors found that alterations in cultural and morphological characters usually accompanied such changes. In the group N streptococcus described here no such correlation was observed.

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REFERENCES

ABD-EL-MALEK, Y. & GIBSON, T. (1948). Studies in the bacteriology of milk. I. The streptococci of milk. *J. Dairy Res.* **15**, 233.

BLISS, E. A. (1937). Studies upon minute haemolytic streptococci. III. Serological differentiation. *J. Bact.* **33**, 625.

BRIGGS, C. A. E. & NEWLAND, L. G. M. (1952). The serological classification of *Streptococcus cremoris*. *J. Dairy Res.* **19**, 160.

DAWSON, M. H., HOBBY, G. L. & OLMSTEAD, M. (1938). Variation in the haemolytic streptococci. *J. infect. Dis.* **62**, 138.

FULLER, A. T. (1938). The formamide method for the extraction of polysaccharide from haemolytic streptococci. *Brit. J. exp. Path.* **19**, 130.

LANCEFIELD, R. C. (1933). A serological differentiation of human and other groups of haemolytic streptococci. *J. exp. Med.* **57**, 571.

MAXTED, W. R. (1949). Occurrence of the M substance of type 28 group A in streptococci of Lancefield groups B, C and G. *J. gen. Microbiol.* **3**, 1.

SEELEMANN, M. & CARSTENS, O. (1951*a*). Die serologische Zugehörigheit des *Sc. bovis* und sog. atypischer Enterokokken zur Gruppe D. *Kieler Milchw. Forsch. Ber.* **3**, 431.

SEELEMANN, M. & CARSTENS, O. (1951*b*). Zur Biologie des *Sc. lactis* und *Sc. cremoris* (Gruppe L). *Milchwissenschaft*, **6**, 231.

SHARPE, M. E. & SHATTOCK, P. M. F. (1952). The serological typing of group D streptococci associated with outbreaks of neonatal diarrhoea. *J. gen. Microbiol.* **6**, 150.

SHATTOCK, P. M. F. (1949). The streptococci of group D; the serological grouping of *Streptococcus bovis* and observations on serologically refractory group D strains. *J. gen. Microbiol.* **3**, 80.

SHATTOCK, P. M. F. & HIRSCH, A. (1947). A liquid medium buffered at pH 9·6 for the differentiation of *Streptococcus faecalis* from *Streptococcus lactis*. *J. Path. Bact.* **59**, 495.

SHATTOCK, P. M. F. & MATTICK, A. T. R. (1943). The serological grouping of *Streptococcus lactis* (group N) and its relationship to *Streptococcus faecalis*. *J. Hyg., Camb.* **43**, 173.

SKADHAUGE, K. (1950). *Studies on Enterococci*. Copenhagen: Einer Munksgaard.

SWARTLING, P. (1951). Biochemical and serological properties of some citric acid fermenting streptococci from milk and dairy products. *J. Dairy Res.* **18**, 256.

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Production, Extraction and Purification of the Haemagglutinin of *Haemophilus pertussis*

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SUMMARY: Most, though not all, freshly isolated strains of *Haemophilus pertussis* contain haemagglutinin. When organisms are grown on solid medium the haemagglutinin is associated with the cell. In liquid cultures the haemagglutinin in the early stages of growth is also associated with the cell but later diffuses into the medium.

Haemagglutinin can be extracted by sodium chloride or sodium acetate from *H. pertussis* grown on solid medium. It can be precipitated from the extracts by methanol in the cold and resuspended in phosphate buffer. Precipitation by methanol gives a considerable degree of purification.

Haemagglutinin deteriorates rapidly on storage even at low temperatures. The rate of deterioration is less with solutions in 50% glycerol. Fowl red blood cells or stromata absorb the haemagglutinin but not the toxin. The sodium chloride extract also contains agglutinogen but the sodium acetate extract does not.

Antihaemagglutinin can be prepared by immunizing rabbits with haemagglutinin either in the form of extracts or bacterial suspensions. The most potent antisera are obtained using as antigen red blood cells saturated with haemagglutinin.

Mice infected either intracerebrally or intranasally with *H. pertussis* cannot be protected either by active immunization with haemagglutinin or by passive immunization with antihaemagglutinin.

It was shown by Keogh & North (1948) that the supernatant fluids from liquid cultures of *Haemophilus pertussis* contained haemagglutinin and were able to produce active immunity in mice to experimental pertussis infection. Although these fluids undoubtedly contained other soluble products of the organism, in addition to haemagglutinin, the authors were of the opinion that the protection induced was related to the haemagglutinin content. This opinion was supported by the results of passive protection tests, in which the protective potency of *H. pertussis* antisera was shown to be a direct function of their antihaemagglutinin content. The work reported in this communication was made to verify these conclusions and to clarify the relationship of haemagglutinin to other recognized antigens of *H. pertussis* such as the agglutinogen and the toxin.

MATERIALS AND METHODS

The haemagglutinin test. A modification of the Keogh & North method was used. Fowl blood was collected aseptically in 3% sodium citrate and the red blood cells (R.B.C.) washed 3 times with 6 vol. of normal saline. For the test 1% R.B.C. suspension in saline was used. Twofold dilutions in saline of the haemagglutinin preparation were made in 2 × 0·5 in. tubes using 0·2 ml. volumes. To each dilution 0·2 ml. 1% R.B.C. suspension was added, the mixtures thoroughly shaken, incubated at 37° and the end-points read after 1 hr. according to the patterns of the sedimented cells using Salk's method (1944). Saline was used throughout as diluent. No improvement in the test was obtained with other diluents such as saline with added cysteine as recommended by Keogh & North, Casamino acids, Lock's solution or 0·2M-phosphate buffer. The temperature of 37° at which haemagglutinin and R.B.C. were incubated was not critical; other temperatures such as 4, 18, 45 and 50° were found to be equally satisfactory and gave the same titres as the tests done at 37°.

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Strains and media. Strains of *H. pertussis* were obtained from the first subculture of the original swabs taken from cases of pertussis. They were maintained in the freshly isolated condition by drying from the frozen state.

The Bordet-Gengou (B.G.) medium used contained 33 % defibrinated horse blood and 1 % Proteose Peptone Difco. No improvement in haemagglutinin production was obtained with medium containing a higher concentration of blood.

The liquid medium was prepared according to the method used by Cohen & Wheeler (1946).

HAEMAGGLUTININ IN CULTURES

Solid medium. Strains were tested for their ability to produce haemagglutinin by titrating suspensions from the 24 hr. growth at 36° on B.G. The suspensions were standardized to contain $10{,}000 \times 10^6$ organisms/ml. against Brown's tubes assuming *H. pertussis* to be equivalent to *H. influenzae*. No difference was found in the haemagglutinin titre of suspensions prepared from 24, 48, 72 or 96 hr. growth. This result was not in agreement with the findings of Ungar (1949), who was probably working with a different type of haemagglutinin system.

Table 1. *The haemagglutinin content of* 120 *strains of* Haemophilus pertussis *in their second or third subculture on* B.G. *medium*

	Strains		
Titre	No.	%	
16	14	11·6	65 % positive
8	22	18·3	
4	10	8·3	
2	32	26·6	
<2	42	25	25 % negative

The titres in the Tables 1–4 and 7 are given as the reciprocal of the highest dilution of suspension or extract which gave complete haemagglutination.

The haemagglutinin titres of 120 strains in their second or third subculture are shown in Table 1. It is evident that there was considerable variation in titre; some strains produced no haemagglutinin while others gave a titre of 1/16. It was also found that the rate of fall in titre with progressive subculture was not the same for all strains. These results are in agreement with those of Standfast (1951*a*, *b*). The ability of strains to produce haemagglutinin was not related to their agglutinogen content, as measured by the agglutinating titre against smooth *H. pertussis* antisera prepared in a rabbit; some strains with high agglutinogen content had no detectable haemagglutinin. Neither was there a correlation between haemagglutinin and the capsulation of the organism (Lawson, 1940), as both haemagglutinin and non-haemagglutinin producing strains proved to be capsulated.

Liquid medium. A typical experiment showing the relation of haemagglutinin production to the growth of *H. pertussis* in liquid medium is shown in Fig. 1. Similar results to this were obtained with three different strains and are in agreement with those of Fisher (1948). Haemagglutinin was found to be present in the bacterial cells during the early stages of growth and thereafter gradually decreased. On the other hand, the supernatant fluid contained in the early stages of growth little or no haemagglutinin but showed increasing titres after the 3rd or 4th days until a maximum was reached on the 8th day. This increase was followed by a rapid decline, probably due to the medium becoming alkaline.

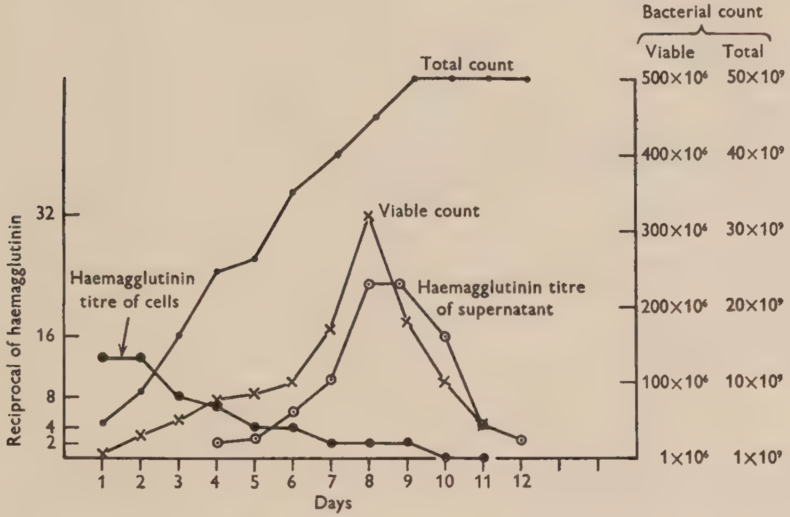

Fig. 1. Relation of haemagglutinin production to growth curves of *H. pertussis*.

Although a number of methods (Table 2) was used in attempts to extract haemagglutinin from *H. pertussis* only two gave extracts with high titre. These are described in detail.

Extraction with NaCl. Strains of high haemagglutinin content were grown on B.G. and harvested after 24 hr. The growth was suspended in 2 M-sodium chloride to a density of 4×10^{10} organisms/ml. and incubated at 37° for 48 hr. The suspension was then centrifuged at 0° at 3000 r.p.m. and the supernatant fluid separated from the deposit. The haemagglutinin titre of the fluid was on the average 1/128. This procedure was arrived at after investigating the effect of extraction with saline concentrations ranging from 0·5 to 2·5 M for periods from 8 to 48 hr. at 0, 18 and 37°. It is evident from Tables 3 and 4 that 2 M-saline for 48 hr. at 37° gave the greatest yield. It was also shown that at temperatures higher than 37° less haemagglutinin was extracted, probably on account of its thermolability. Washing the organisms with saline or water, or grinding the dry organisms in a mortar before extraction did not increase the yield of haemagglutinin in the extract.

Table 2. *Methods used in attempts to extract haemagglutinin from* H. pertussis

Method	Author	Average haemagglutinin titre of extract
Water	Flosdorf & Kimball (1940)	<2
0·85% saline	Katasampes *et al.* (1942)	<2
NaOH	Miller (1934)	<2
Urea	Hink & Johnson (1947)	<2
HCl	Smolens & Mudd (1943)	8
Freezing and thawing	Evans & Maitland (1937)	8
Grinding with alumina	McIlwain (1948)	<2
2 M-NaCl	Murray (1929)	128
M-sodium acetate	Masry	128–256

Table 3. *Titre of supernatant fluids of* H. pertussis *after extraction with* NaCl *at* 37°

Time of extraction (hr.)	Salt concentration				
	0·5 M	1·0 M	1·5 M	2·0 M	2·5 M
8	<2	<2	<2	<2	<2
18	<2	4	4	16	8
24	2	4	8	64	16
48	4	4	32	128	32

Table 4. *Titre of supernatant fluids of* H. pertussis *after extraction with 2 M-sodium chloride*

Temperature of extraction	Time of extraction (hr.)	Titre
0°	8	<2
	18	8
	24	32
	48	64
18°	8	<2
	18	8
	24	32
	48	128
37°	8	<2
	18	8
	24	64
	48	256

Extraction with sodium acetate and precipitation with methanol. The extraction of haemagglutinin by sodium acetate was investigated by varying the conditions of extraction as in the sodium chloride extraction. The most potent extracts were obtained with M-sodium acetate at 37° for 48 hr. with an optimal density of 8×10^{10} organisms/ml. The titre of haemagglutinin in the extract was on the average 1/128 to 1/256. Haemagglutinin could be extracted from organisms which had been washed with saline or distilled water, and this was a point of practical importance as large-scale production involved the harvesting of organisms from Roux bottles by flooding the growth off the surface of the medium.

Haemagglutinin was precipitated from the sodium acetate extracts by methanol, a method used by Pillemer (1946) and Pillemer, Toll & Badger (1947) for the purification of diphtheria and tetanus toxins, and by Robbins & Pillemer (1950) for precipitating the protective fraction from *H. pertussis*. The sodium acetate extract was cooled to −3° and the pH adjusted to be slightly acid. Methanol chilled to −18° was added slowly (about 2 ml./min.) with constant stirring, and the temperature of the mixture maintained at −5° throughout. The final concentration of methanol was 40% by volume. The mixtures were allowed to remain at −10° overnight and were then centrifuged at −10° for 1 hr. at 5000 r.p.m. The supernatant fluid was removed and the precipitate resuspended in distilled water at 2° and dried from the frozen state. The dry material was readily soluble in 0·5 M-phosphate buffer at pH 6·8.

This method brought about a considerable degree of purification of the haemagglutinin. Before methanol precipitation, extracts with a titre of 1/128 to 1/256 contained an average of 0·7 mg. N/ml., and after precipitation solutions of the precipitates with the same titre contained only 0·02–0·03 mg. N/ml.

PROPERTIES OF HAEMAGGLUTININ

Antigenicity of haemagglutinin. Rabbits immunized with haemagglutinin, in the form of either extract or bacterial suspension, produced antihaemagglutinin, the potency of which was titrated by determining the dilution of antiserum necessary to neutralize 10 minimal haemagglutinin units (m.h.u.). One haemagglutinin unit was taken as the minimal amount of a solution of haemagglutinin which produced complete agglutination of 0·2 ml. of 0·5% suspension of fowl R.B.C. It was immaterial which haemagglutinin preparation was used for the titration of antisera. Titrations made with the supernatants from 8-day fluid cultures, sodium chloride extracts, sodium acetate extracts and solutions of the methanol precipitate, gave the same titre of antihaemagglutinin for any one serum. The haemagglutinin in all four preparations therefore appeared to be antigenically the same.

Agglutinogen content of extracts. The sodium chloride and sodium acetate extracts were tested for agglutinogen by a method similar to that used by Flosdorf & Kimball (1940). *H. pertussis* antiserum with high agglutinin titre was absorbed with extract and the extent of the absorption determined by titrating the agglutinins before and after absorption. Each of five different antisera were absorbed with each of the two extracts in varying dilutions, and typical results with one serum are given in Table 5. The sodium chloride extract when undiluted decreased the agglutinin titre of the serum from 1/25,600 to 1/3200 and even with extract diluted ½ and ¼ the titre was decreased to 1/6400. On the other hand, the sodium acetate extract even undiluted had no effect on the agglutinin titre. This result indicated that the sodium chloride extract contained agglutinogen but that the sodium acetate extract did not. Support for these findings was obtained by immunizing rabbits with each extract; antisera prepared against the sodium chloride extract had an agglutinating titre of 1/4000 but no agglutinins were detected in antisera prepared against the sodium acetate extract.

Table 5. *Adsorption of agglutinin from* H. pertussis *antiserum by haemagglutinin extracts*

Absorbent		Dilution of antibacterial serum				
Type	Concentration	3,200	6,400	12,800	25,600	51,200
	None	++	++	++	±	0
Sodium chloride extract	Undiluted	±	0	0	0	0
	1 : 2	+	±	0	0	0
	1 : 4	+	+	0	0	0
	1 : 8	++	++	+	±	0
	1 : 16	++	++	++	±	0
Sodium acetate extract	Undiluted	++	++	+	±	0
	1 : 2	++	++	++	0	0
	1 : 4	++	++	+	±	0
	1 : 8	++	++	++	+	0
	1 : 16	++	++	++	±	0

++ to 0 = degree of agglutination.

Toxicity of extracts. The toxicity of three haemagglutinin preparations was tested: the sodium chloride extract, the sodium acetate extract and the methanol precipitated material. Tests were made by both the intradermal injection of rabbits and the intravenous injection of mice using solutions each with a haemagglutinin titre of 1/128. Each extract was toxic. The intradermal injection produced necrotic lesions, similar to those described by Evans & Maitland (1937) for the thermolabile toxin of *H. pertussis*, and intravenous injection proved lethal. The potency of the toxin in the extracts was not however similar and, as each extract had the same haemagglutinin titre, it was concluded that the toxic and haemagglutinin factors were different. This conclusion was confirmed, for it was shown that 10% washed R.B.C. when mixed with extracts and incubated for 1 hr. at 37° completely absorbed the haemagglutinin but not the toxin. A similar separation was achieved using stromata.

Thermolability. Haemagglutinin reconstituted in 0·2 M-phosphate buffer pH 6·8 to give a titre of 1/128 was titrated after it had been incubated at various temperatures for different periods. Complete loss occurred within a few minutes at 60°, after 72 hr. at 37° and after 4 days at room temperature. Storage at 4° caused the titre to fall 25% in 2 weeks and after 35 days no haemagglutinin was detected. Storage at −10° did not prevent deterioration; in 60 days the activity was completely lost.

Effect of preservatives. The rate of deterioration of haemagglutinin was not influenced by the addition of either 0·3% formalin, 0·5% phenol or 1/10,000 thiomersalate. Glycerol, however, was found to retard the rate of deterioration when added in a concentration of 50 %; lower concentrations were not so effective.

Filtration. Haemagglutinin was almost completely absorbed during filtration through Seitz filters. Treatment of the filter pad with 50 % rabbit serum and phosphate buffer (Evans & Maitland, 1937) before filtration did not reduce the absorptive capacity of the filter for haemagglutinin. Filtration through Gradocol filters, however, proved to be successful as only a slight loss in activity occurred.

HAEMAGGLUTININ IN RELATION TO IMMUNITY

Active immunization of mice. Mice were immunized intraperitoneally with haemagglutinin preparations and challenged either intracerebrally or intranasally with living *H. pertussis.* Six different preparations of methanol-precipitated haemagglutinin were tested. They were first dissolved in phosphate buffer to give a titre of 1/128 and then absorbed on to R.B.C. This adsorption was repeated with fresh batches of extracts until the cells were saturated with haemagglutinin and unable to absorb further amounts. A 10 % suspension of the R.B.C. was then made and was calculated from tests on the haemagglutinin solutions before and after absorption to contain about 4000 m.h.u./ml. Three groups of mice, fifteen in each group, were injected intraperitoneally with the R.B.C. antigen. The dose for the first group was 1·0 ml., for the second 0·75 ml. and for the third 0·5 ml. The injections were repeated 5 days later, and after 10 days each mouse was challenged intracerebrally according to the method of Kendrick, Updyke & Eldering (1949) with approximately 200 LD 50 of living *H. pertussis*. The results are given in Table 6, and it is evident that little or no protection was produced by any of the preparations when compared with that given by a control bacterial vaccine.

Similar results were also obtained (Table 6) when the intranasal challenge route was used.

Passive immunization of mice. Antihaemagglutinin was prepared in rabbits using haemagglutinin absorbed on to R.B.C. The antiserum which had an antihaemagglutinin titre of 1/1200 was tested for its ability to protect mice against pertussis infection. A mixture of equal parts of undiluted antiserum and a suspension of *H. pertussis* was made to contain 2500×10^6 organisms/ml. The mixture was inoculated intranasally into mice (Evans, 1944), and each animal received approximately 0·05 ml. which contained 100×10^6 organisms equivalent to 100 LD 50. Results are given in Table 7 and it is evident that the antiserum showed little or no protective property. In other experiments (Table 7) the antiserum was given intraperitoneally 4 hr. before the mice were challenged by intracerebral injection. Again there was little evidence that the antihaemagglutinin serum was able to protect against infection. In this experiment antiserum prepared against whole organisms was also tested and gave substantial protection.

Table 6. *Active immunization of mice with purified haemagglutinins adsorbed on erythrocytes*

	Dose		
Haemagglutinin batch no.	2 × 1 ml.	2 × 0·75 ml.	2 × 0·5 ml.
	No. survivors/total mice		
	Against intracerebral challenge with *H. pertussis*		
1	3/12	4/15	1/11
2	2/11	2/15	3/15
3	2/11	2/11	1/13
4	3/15	0/11	2/14
5	1/15	2/15	1/15
6	4/15	3/14	2/11
Total	15/79	13/81	10/79
Control bacterial vaccine*	51/80	30/80	19/76
Control unimmunized mice	3/88		
	Against intranasal challenge with *H. pertussis*		
1	3/15	2/15	2/12
2	2/13	3/15	3/14
3	2/15	1/15	3/15
4	2/15	4/15	3/15
5	3/14	0/15	5/15
6	4/15	1/13	0/14
Total	16/87	11/88	15/85
Control bacterial vaccine*	44/77	31/84	16/81
Control unimmunized mice	12/90		

* Doses, control bacterial vaccine: 1 × 0·2 ml., 1 × 0·04 ml., 1 × 0·008 ml. of a vaccine containing $10{,}000 \times 10^6$/ml.

Table 7. *Passive protection of mice with sera prepared in rabbits against purified haemagglutinins adsorbed on erythrocytes*

Serum no.	Anti-haemagglutinin titre	No. protected/total mice	Average time to death (days)
		Serum and challenge dose of *H. pertussis* mixed and given intranasally	
1	1200	1/13	4·58
2	600	1/14	5·69
3	1200	3/10, 2/15, 3/15 = 8/40	5·15
4*	< 2	2/10, 2/15, 2/15 = 6/40	5·84
5†		9/10, 13/15, 13/15 = 35/40	
		Serum given intraperitoneally 4 hr. before intracerebral challenge	
6	1400	3/7, 2/10, 1/10 = 6/27	7·3
7	600	3/8, 0/10, 3/10 = 6/28	7·1
4*	< 2	0/10, 2/9, 0/10 = 2/29	6·1
5†	10	9/10, 10/10, 5/10 = 24/30	10·4

* Normal rabbit serum.

† Immune rabbit serum prepared against living *H. pertussis* cells.

CONCLUSIONS

These experiments have shown that the haemagglutinin of *H. pertussis* can be extracted from the bacillus by sodium acetate and concentrated by absorption on to fowl erythrocytes. This preparation was free from the bacterial agglutinogen and toxin, and thus it was possible to study the protective properties of haemagglutinin alone. It is evident from the results that the purified haemagglutinin was unable to induce an active immunity to experimental pertussis infection in mice and that antiserum with a high content of antihaemagglutinin gave no passive protection. These results suggest that although haemagglutinin is antigenically active it is not of importance in the control of experimental infection.

These results are not, however, in agreement with those of Keogh & North who expressed the opinion that haemagglutinin was able to induce protection against experimental infection. The antigen they used in their experiments was not, however, free from other antigens of *H. pertussis* and did in fact contain the bacterial agglutinogen.

These results are in agreement with those recently obtained by Pillemer (1950), who has prepared a protective antigen from ultrasonic extracts of *H. pertussis* and says that this antigen did not contain any haemagglutinin.

This work formed part of a thesis approved by the University of London for the award of a Ph.D. Degree.

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REFERENCES

Cohen, S. M. & Wheeler, M. W. (1946). Pertussis vaccine prepared with phase I cultures grown in fluid medium. *Amer. J. publ. Hlth*, **36**, 371.

Evans, D. G. (1944). The protective properties of pertussis anti-sera in experimental infection. *J. Path. Bact.* **56**, 49.

Evans, D. G. & Maitland, H. B. (1937). The preparation of the toxin of *H. pertussis*: its properties and relation to immunity. *J. Path. Bact.* **45**, 715.

Fisher, S. (1948). The behaviour of *H. pertussis* in casein hydrolysate broth. *Aust. J. exp. Biol. med. Sci.* **26**, 299.

Flosdorf, E. W. & Kimball, A. C. (1940). Comparison of various physical means of liberation of agglutinogen of *H. pertussis* phase I. *J. Immunol.* **39**, 287.

Hink, J. H. & Johnson, F. F. (1947). Preparation of an antigen mixture of *H. pertussis* by selective denaturation. *J. Immunol.* **57**, 323.

Katasampes, C. P., Brooks, A. M. & Bradford, W. L. (1942). Toxicity of washings from *Hemophilus pertussis* for mice. *Proc. Soc. exp. Biol., N.Y.* **49**, 615.

Kendrick, P. L., Updyke, E. L. & Eldering, G. (1949). Comparison of pertussis cultures by mouse protection and virulence tests. *Amer. J. publ. Hlth*, **39**, 179.

Keogh, E. V. & North, E. A. (1948). The haemagglutinin of *H. pertussis*. 1. Haemagglutinin as a protective antigen in experimental murine pertussis. *Aust. J. exp. Biol. med. Sci.* **26**, 315.

Lawson, G. M. (1940). Modified technique for staining capsules of *Haemophilus pertussis*. *J. Lab. clin. Med.* **25**, 435.

McIlwain, H. (1948). Preparation of cell free bacterial extracts with powdered alumina. *J. gen. Microbiol.* **2**, 288.

Miller, J. J. (1934). Experimental observations on the antigenic potency of *H. pertussis* extracts. *J. Immunol.* **26**, 247.

Murray, E. G. D. (1929). The *Meningococcus. Spec. Rep. Ser. med. Res. Coun., Lond.* no. 124.

Pillemer, L. (1946). The immunochemistry of toxins and toxoids. 1. The solubility and precipitation of tetanol toxin and toxoid in methanol-water mixture under controlled conditions of pH, ionic strength and temperature. *J. Immunol.* **53**, 237.

Pillemer, L. (1950). Adsorption of protective antigen of *H. pertussis* on human red cell stromata. *Proc. Soc. exp. Biol., N.Y.* **75**, 704.

Pillemer, L., Toll, D. & Badger, S. J. (1947). Immunochemistry of toxins and toxoids. III. Isolation and characterisation of diphtherial toxoid. *J. biol. Chem.* **170**, 571.

Robbins, K. C. & Pillemer, L. (1950). The separation of a protective antigen from a toxin-producing strain of *Hemophilus pertussis. J. Immunol.* **65**, 393.

Salk, J. E. (1944). A simplified procedure for titrating haemagglutinating capacity of influenza virus and the corresponding antibody. *J. Immunol.* **49**, 87.

Smolens, J. & Mudd, S. (1943). Agglutinogen of *Haemophilus pertussis*, Phase 1, for skin-testing. Theoretical consideration, and a simple method of preparation. *J. Immunol.* **47**, 155.

Standfast, A. F. B. (1951*a*). The virulence of *Haemophilus pertussis* for mice by the intranasal route. *J. gen. Microbiol.* **5**, 250.

Standfast, A. F. B. (1951*b*). The phase 1 of *Haemophilus pertussis. J. gen. Microbiol.* **5**, 531.

Ungar, J. (1949). Agglutination of red blood cells by *H. pertussis. J. Path. Bact.* **61**, 140.

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A Comparison of Methods for Removing Trace Metals from Microbiological Media

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SUMMARY: Thirty-eight methods or modifications of methods for removing trace metals from nutrient media were tested, using *Aspergillus niger* as test organism to indicate deficiencies.

The most marked symptoms of Zn and Fe deficiencies were obtained after treatment by the Al_2O_3 or dithizone + oxine methods, of Cu and Mn deficiencies by the Al_2O_3 method, and of Mo deficiency by Nicholas's method which involves co-precipitation with sulphides of Cu and Pb. Four strains of *A. niger* differed in their trace element requirements. Shake cultures of *A. niger* gave less growth in complete medium than did surface cultures, while in deficient media this was reversed. Sucrose peptone and broth media were only partially purified by the Al_2O_3 treatment. Deficiencies of Fe, Zn and Cu were obtained for *Chlorella vulgaris*, and of Fe and Zn for *Torulopsis utilis*, as detected by growth response. Apart from decrease of growth, trace metal deficiency symptoms in *Penicillium roqueforti* were not very obvious.

This paper summarizes work undertaken to compare the relative merits of most known methods for removing Fe, Zn, Cu, Mn and Mo from culture media, including methods and modifications devised by the present authors.

Foster (1949) outlined many methods for freeing media from trace metals, without deciding which are the most efficient. Steinberg (1950) compared the relative merits of a number of techniques, only to find that his method, devised in 1919, which uses $CaCO_3$ as an adsorbent, was superior to many advocated more recently.

The various methods available can be divided into two broad groups: (*a*) those which employ a solid such as charcoal or $CaCO_3$ for adsorbing trace elements, the solid adsorbent being subsequently removed by filtration; (*b*) those which employ chelating agents such as diphenylthiocarbazone (dithizone) or 8-hydroxyquinoline (oxine), which combine with metals to form water-insoluble complexes that can be removed by extraction with organic solvents such as ether or carbon tetrachloride. In a few methods, both techniques are combined.

MATERIALS AND METHODS

Test organisms. Several strains of *Aspergillus niger* were tested: 540 and 543R, used for the commercial production of citric acid; M used by Mulder (1948) and 'Gerretsen' used by Gerretsen (1948) for determining the availability of trace elements in Dutch soils. In addition an alga, *Chlorella vulgaris*, and a yeast, *Torulopsis utilis*, were used. All stock cultures were maintained in tubes of liquid media treated with Al_2O_3 to remove trace metals, the treatment still leaving enough trace metals for maintenance in this way. Inocula were prepared by suspending a loopful of spores of *A. niger*, or cells of *C. vulgaris* and *T. utilis*, in 10 ml. of water twice distilled in glass, and seeding each flask with one drop from a sterile, acid-cleaned pipette.

Media. The basal medium for *A. niger* and *T. utilis* contained 50 g. sucrose (commercial), 5 g. KNO_3 (A.R.), 2·5 g. K_2HPO_4 (C.P.), and 0·5 g. $MgSO_4.7H_2O$, made up to 1 l. with water from a tinned copper still. Nicholas's (1950) medium was used when testing his method for removing Mo. In order to decide whether the purification methods were effective, known amounts of trace metals were deliberately added to the medium before treatment. After purification a complete medium was prepared by adding recrystallized salts in the following amounts to 1 l.: 200 μg. Fe (as $FeSO_4.7H_2O$); 180 μg. Zn (as $ZnSO_4.7H_2O$); 40 μg. Cu (as $CuSO_4.5H_2O$); 20 μg. Mn (as $MnSO_4.4H_2O$); 10 μg. Mo (as $(NH_4)_2MoO_4$); 10 μg. B (as H_3BO_3). Trace metal deficient media were prepared by omitting the element in question.

C. vulgaris was grown in the following medium: 2 g. NH_4NO_3 (A.R.), 1 g. K_2HPO_4 (C.P.), 0·25 g. $MgSO_4.7H_2O$ (A.R.), made up with water from a tinned copper still to 1 l. Trace elements were deliberately added to the unpurified medium as above. After treatment trace metals, as the salts given above, were added where necessary in the following amounts, to 1 l. of medium: 400 μg. Fe; 140 μg. Zn; 2 μg. Cu; 110 μg. Mn; 50 μg. Mo; 50 μg. B.

Attempts were also made to remove trace elements from more complex media, viz. sucrose (5%) peptone water, and sucrose (5%) ox heart broth, using the Al_2O_3 method.

All purified media were adjusted to pH 7 before inoculation by adding either redistilled NH_4OH or 6N-HCl, except where otherwise stated. All glassware was cleaned by immersion in *aqua regia* overnight, followed by three rinses with water from a tinned copper still and three rinses with water twice distilled from glass.

Methods of cultivation. In static culture, *A. niger* was grown in 25 ml. of medium in 100 ml. Pyrex conical flasks, covered with inverted glass jars, or in Petri dishes (10 cm. diameter), and incubated at 30° for 7 days when maximum growth was achieved. The mycelium was then harvested, dried overnight at 40°, and weighed on a torsion balance. Shaken cultures were grown for 7 days at 30° in 60 ml. medium contained in 1 l. reagent bottles covered with beakers, and shaken at the rate of 172 shakes/min. through a distance of 2 in.

T. utilis was grown in static culture as above and incubated at 30° for 14 days; amount of growth was estimated by turbidity measurements.

C. vulgaris was grown in static and shaken cultures at 32°, illuminated by twelve 60 W. incandescent electric lamps held at a height of 18 in. above the cultures. Incubation was continued for 14 days and the growth was estimated by turbidity measurements.

METHODS USED FOR REMOVING TRACE METALS

Adsorption methods

(1) Steinberg's (1919) method using $CaCO_3$ and autoclaving once.

(2) Steinberg's $CaCO_3$ method modified by bubbling H_2S through the medium before autoclaving.

(3) Bortel's (1927) method using Norite charcoal.

(4) Mulder's (1948) method using Norite charcoal and NH_4HS.

(5) Al_2O_3 method. Five g. Al_2O_3 (British Drug Houses Ltd. chromatographic adsorption analysis material) were added to 1 l. of medium (pH 7·5) in a 1·5 l. flask, which was shaken and autoclaved at 10 lb./sq.in. for 20 min. On removal from the autoclave the hot contents were swirled to wet the inside of the flask. After standing overnight, the medium was sucked through an acid-treated, sintered-glass Buchner funnel connected by glass joints to a Pyrex filter flask. The filtrate was re-adjusted to pH 7, and autoclaved.

The Al_2O_3 procedure was carried out at various pH values between 3·5 and 8·5, the most marked deficiency symptoms being obtained after using Al_2O_3 at pH 7·5. Acid-washed chromatographic Al_2O_3 gave no more marked deficiency symptoms, while Al_2O_3 (A.R.) was less effective.

(6) $Al_2O_3 + H_2S$ method. H_2S was bubbled through 1 l. of medium at pH 7 for 30 min., 5 g. chromatographic Al_2O_3 added, and the medium autoclaved at 10 lb./sq.in. for 20 min. After filtration the pH value of the filtrate was adjusted to 3, the medium boiled and aerated to eliminate H_2S, re-adjusted to pH 7, then autoclaved.

(7) $Al_2O_3 + CaCO_3$ method. Two g. of chromatographic $Al_2O_3 + 5$ g. $CaCO_3$ (A.R.) were added to 1 l. of medium at pH 8, which was autoclaved as before, then filtered, the reaction of the precipitate adjusted to pH 7, then autoclaved.

(8) Magnesium phosphate method. Magnesium phosphate was precipitated by adding 0·25 g. $MgSO_4.7H_2O$/l. medium at pH 8, autoclaving and filtering.

(9) Other adsorbents. Unsuccessful attempts were made to improve on the results obtained with Al_2O_3 by boiling various samples of media at pH 8 with MnO_2, Fe_2O_3, ionic exchange resins, humic acid, casein, albumin, bentonite, kaolin, or various soils.

(10) Nicholas's (1950) method for removal of Mo, which involves co-precipitation of sulphides of Cu, Pb and Mo at pH 3 and readjusting to pH 2 prior to inoculation, as recommended by Nicholas. Nicholas's method often gave poor growth, probably because of residual traces of toxic sulphur compounds. Unsuccessful attempts were made to improve Nicholas's method by absorbing the Mo from boiling medium at pH 3 on to Al_2O_3 (with or without H_2S), washed sulphides of Cu and Pb, flowers of sulphur moistened with ethanol, and colloidal sulphur formed by adding acidified sodium thiosulphate.

Chelation methods

(11) Diphenylthiocarbazone (dithizone). A purified solution of dithizone (0·02 %, w/v) in redistilled CCl_4 was prepared by the method of Piper (1944), and 100 ml. of this solution were added to 2 l. of medium adjusted to pH 9

with redistilled NH_4OH. The mixture was shaken for 4 min. in a 2·5 l. separating funnel on a reciprocal shaker at the rate of 172 shakes/min. through a distance of 2 in. The CCl_4 layer was run off and the extraction repeated until 5 ml. of medium gave no pink colour when shaken with 1 drop of ammoniacal dithizone in CCl_4. At least three extractions were necessary. Dithizone was removed from the medium at pH values of 9, 7 and 4 by repeated washing with redistilled CCl_4, three washings at each pH usually proving satisfactory. The medium was then boiled to eliminate CCl_4, re-adjusted to pH 7, and autoclaved.

(12) 8-Hydroxyquinoline (oxine). The following modification of Waring & Werkman's (1942) method was used. Two l. of medium were shaken as above with 50 ml. of a solution of oxine (0·5 %, w/v) in redistilled CCl_4 at pH 9. The CCl_4 layer was run off, and the extraction repeated at least three times. The medium was then washed at pH 9 and 7 with redistilled CCl_4, and boiled to remove CCl_4, then autoclaved.

(13) Dithizone + oxine. The batch of medium was repeatedly shaken as above, first at pH 9 with oxine in CCl_4, and then at pH 7 with dithizone in CCl_4. The chelating agents left in the medium were removed by repeated extractions at pH 9 with redistilled CCl_4 after the oxine extraction, and at pH 6 and 4 after the dithizone extraction. The medium was boiled to remove CCl_4, adjusted to pH 7 and autoclaved. This procedure was varied in several ways to determine the best conditions for the removal of trace metals, e.g. by adding the dithizone and oxine solutions together to the medium, and by making extractions at various pH values, viz. at 9 only, or at 9, 8, 7, 6 and 5 in succession. Deficiency symptoms were not more marked after successive extractions at pH values from 9 to 5 than at 9 only. However, dithizone was not removed completely from the aqueous layer after extraction at pH 9 with CCl_4, and yellow oxidation products were formed; dithizone was therefore used at pH 7, at which pH value it is equally effective as a chelating agent. Deficiency symptoms were not more marked after extracting 10 % solutions of sucrose and salts separately and making up to volume with water twice distilled in glass.

(14) Nicholas's (1950) method for removal of Mn, which uses sodium diethyldithiocarbonate (NaDDC) at pH 3·4, surplus chelating agent being removed by Cu, and excess Cu by H_2S.

(15) Nicholas's (1950) method for removal of Mo by co-precipitation with Fe in the presence of 8-hydroxyquinoline.

RESULTS

Results with Aspergillus niger *as test organism*

The extent of removal of each trace metal was estimated by the diminution of growth below that obtained in a complete medium. However, in addition to this decrease in weight of mycelium, there were characteristic changes in the macroscopic and microscopic appearance of static cultures of the organism brought about by each specific deficiency (see Pl. 1). In Fe deficient cultures.

mycelial growth was sparse with a few small black spore heads. With Zn deficiency, the mat was very thin, mainly submerged, and dotted with large black spore heads. Cu deficiency did not cause such a marked decrease in mycelium, but the spores lost their black pigment and with extreme deficiency spores were absent. In Mn deficiency, sporulation was almost absent and there was a marked decrease in weight of hyphae. Discrete papery colonies were formed instead of a continuous mat, giving a 'cauliflower' appearance, or in extreme deficiencies looking like 'puffed wheat'. In Mo deficient cultures, the mycelium was sparse, submerged, and almost devoid of spores.

Table 1. *Comparison of methods for removing trace metals from sucrose* (5 %) *minerals medium using, as test organisms, two strains of* Aspergillus niger, *grown in static culture*

Method for removing trace metals	Strain of *A. niger* used	No. of tests	Dry weight of mycelium in complete medium (g./25 ml.)	Weight of mycelium in deficient media, expressed as percentage of that in complete media				
				Metal deficiency				
				Fe	Zn	Cu	Mn	Mo
(1) $CaCO_3$	M	7	0·39	27	7·5	58·5	100	80
(2) $CaCO_3+H_2S$	M	2	0·41	29	9·5	74	100	100
(3) Norite	543 R	1	0·20	45	4·5	100	100	100
(4) Norite + NH_4HS	543 R	1	0·11	21	3·5	72	86·5	100
(5) Al_2O_3	M	17	0·43	5	2	33	36	42
(6) $Al_2O_3+H_2S$	M	1	0·38	30	10·5	100	100	—
(7) $Al_2O_3+CaCO_3$	M	1	0·37	27	7·5	57	100	100
(8) Magnesium phosphate	M	1	0·40	10·5	10·5	65	100	74
(10) Nicholas (removal Mo)	M	5	0·36	—	—	—	—	11
(11) Dithizone	M	1	0·39	33	1·5	45·5	73·5	84·5
(11) Dithizone	543 R	2	0·30	65·5	4	54·5	80	21·5
(12) Oxine	543 R	2	0·29	11·5	2·5	55	70·5	58
(13) Dithizone + oxine	M	7	0·51	5	2	49	55	—
(13) Dithizone + oxine	543 R	5	0·29	3	1	51·5	50·5	65
(14) Nicholas (removal Mn)	M	1	0·52	25·5	4	100	63	28
(15) Nicholas (removal Mo)	M	1	0·53	—	—	—	—	66·5

Metal deficiencies revealed in static cultures. Table 1 shows the weights of mycelia of *A. niger* (strains M and 543 R) grown in complete medium, and the weights in deficient media expressed as a percentage of that in complete medium. The most marked Fe deficiency symptoms were obtained with strain M after Al_2O_3 (method no. 5) and with strains M and 543 R after dithizone + oxine treatments (no. 13), which gave mycelial yields of less than 5 % of the maximum. Methods involving magnesium phosphate (no. 8), using strain M, or oxine (no. 12), using strain 543RR, were the next most effective, with yields in Fe-deficient media of 10·5 and 11·5 % respectively; other treatments gave yields of 20–30 % of the maximum, with the exception of the Norite treatment (no. 3) which had a yield of 45 %, and dithizone (no. 11), which gave a 33 % yield with M strain and 65 % with 543 R.

Zn was most effectively removed by Al_2O_3 (no. 5), dithizone (no. 11), and dithizone + oxine (no. 13), with yields of strain M grown in deficient media of 2, 1·5 and 2 % of the maximum respectively. With strain 543R most pronounced Zn deficiencies were achieved with dithizone + oxine (no. 13), dithizone (no. 11) and Norite charcoal + NH_4HS (no. 4). By all treatments, the mass of growth was decreased to one-tenth or less of that in complete media. Zn is evidently comparatively easy to remove, even when present originally in fairly large amounts.

The most marked Cu deficiency with strain M was obtained after Al_2O_3 treatment (no. 5); good removal of Cu was also obtained by dithizone (no. 11) and by dithizone + oxine (no. 13). Of the methods used to induce Cu deficiencies in strain 543R methods 11, 12 and 13 were most successful. Even where no reduction in weight of mycelium occurred, there was a decrease in pigmentation.

Using strain M, Mn was best removed by the Al_2O_3 treatment (no. 5), and with strains M and 543R dithizone + oxine (no. 13) was fairly effective. Even where there was no decrease in mass of growth, all methods of treatment caused some 'cauliflower' or 'puffed wheat' appearance, characteristic of Mn-deficient mycelium.

Mo deficiencies were difficult to obtain. Nicholas's adsorption (1950) method (no. 10 above) was most effective; medium so treated gave a yield with strain M of only 11 % of the maximum. Dithizone treatment (no. 11) gave 21·5 % of the maximum yield of 543R in one case, but results with this method were erratic. Nicholas's (1950) method which uses NaDDC, and was designed for removing Mn (no. 14) was comparatively efficient for removal of Mo; yields with strain M of about 28 % of the maximum were obtained by its use. Al_2O_3 (no. 5) and oxine (no. 12) gave 42 and 58 % respectively, of maximum yield obtained from strains M and 543R. Most other treatments gave no decrease in growth or visible change in macroscopic appearance.

From these results, it is concluded that method no. 5, using chromatographic Al_2O_3 gives better removal of Cu and Mn than any other method tested, and it is as effective as the dithizone + oxine method (no. 13) for removing Zn and Fe, and not nearly so laborious. Nicholas's (1950) method involving co-precipitation with sulphides of Cu, Pb and Mo (no. 10) was the most effective for removing Mo. In our experience, $CaCO_3$ treatment (no. 1) is inferior to our Al_2O_3 method (no. 5), and only slightly better than adsorption by magnesium phosphate (no. 8). Methods involving Norite charcoal, H_2S or oxine, often gave poor growth in the treated medium, subsequently supplemented with essential trace metals, due to the introduction of toxic substances which were not completely removed.

Response of different strains of Aspergillus niger *in various deficient media.* Different strains of *A. niger* were grown in 100 ml. conical flasks in batches of medium of the composition given above, but with only 2 % sucrose, which had been rendered deficient in trace metals by various methods. The results are shown in Table 2, where the means of duplicates are quoted.

In a medium purified with dithizone (no. 11), strain 543R produced more marked Fe, Zn, Cu and Mo deficiency symptoms than strain 540, while the reverse was true for Mn deficiency symptoms. In an oxine purified (no. 12) medium strain 540 showed the most marked deficiency symptoms of Fe, Zn, Mn and Mo, but Cu deficiency was greatest with strain 543R. When the medium was purified with dithizone + oxine (no. 13) and four strains were tested, symptoms of Fe deficiency were most marked with strain 543R, of Zn deficiency with Gerretsen's strain, of Cu deficiency with strains M and 'Gerretsen', of Mn with strain 540, of Mo with strains 540 and 543R.

Table 2. *Growth of strains of* Aspergillus niger *in static culture on sucrose* (2 %) *minerals medium treated by various methods to remove trace metals*

Method for removing trace metals	Strain of *A. niger* used	Dry weight of mycelium in complete medium (g./25 ml.)	Weight of mycelium in deficient media, expressed as percentage of that in complete media Metal deficiency				
			Fe	Zn	Cu	Mn	Mo
Dithizone (no. 11)	540	0·10	80	53	100	72·5	100
	543R	0·14	74	22	63	100	21·5
Oxine (no. 12)	540	0·09	8	17	90	50·5	75
	543R	0·12	17	56·5	76·5	100	100
Dithizone + oxine	540	0·09	8	17	90	50·5	75
	543R	0·14	0	9	73	87	75
No. 13	Gerretsen	0·19	25	6	61	60	100
	M	0·17	20·5	8	60	55	100

It is therefore concluded that Fe deficiency is best detected with strain 543R or 540; the 543R, 'Gerretsen' and M strains are probably equally sensitive to Zn deficiency, and 'Gerretsen' and M to Cu deficiency. Strain 540 is most sensitive to lack of Mn, and strain 543R the most sensitive to Mo deficiency, though response to Mo would be best tested in a medium purified by Nicholas's method (no. 10).

Metal deficiencies in complex media. Sucrose (5 %) broth and peptone were treated by the Al_2O_3 method (no. 5). The mycelial weights of *A. niger*, strain M, obtained on these media after the addition of optimum amounts of trace elements were 0·50 and 0·51 g. respectively, while the yields from the deficient media were 41 and 12 % of the maximum respectively. Thus only partial removal of trace elements was achieved, but it was sufficient to show the usefulness of the method when applied to complex media.

Metal deficiencies revealed in shaken cultures. The results from three experiments showed that in complete medium, the weight of mycelium from shaken cultures was less than 62 % of that from surface growth, whereas in deficient media, the yields from shaken cultures were greater than from the corresponding static ones. In a typical shaken culture experiment in which *A. niger*, strain M, was grown in 60 ml. of medium treated by the Al_2O_3 method (no. 5), 0·67 g. mycelium was obtained in the presence of optimum concentrations of trace elements. In batches of this medium deficient in Fe, Zn, Cu, Mn or Mo the yields, as percentage of maximum, were: 29·0, 9·8, 94·0, 100 and 108 respectively. Pl. 2 illustrates the macroscopic appearance of the growth obtained. Static cultures gave 1·07 g. of mycelium from complete medium and yields, as percentage of maximum, of 9·1, 1·3, 59·0, 46·0 and 79·0, in media deficient in Fe, Zn, Cu, Mn and Mo respectively.

Results with other organisms

Chlorella vulgaris. Media were purified by $CaCO_3$, with (no. 2) and without (no. 1) H_2S, by dithizone + oxine (no. 13), by dithizone (no. 11), and by oxine (no. 12). In static and in shaken cultures growth of the alga was poorest in the Fe-deficient medium, almost white cells being produced. The growth of cells in the Zn- and Cu-deficient media was only slightly greater, and the cells were green. Media treated to remove Mn or Mo did not give smaller yields or alter the colour of the cells as compared with the results obtained in complete medium.

Torulopsis utilis. Only Fe and Zn deficiencies, obtained by using media purified with dithizone + oxine (no. 13), or with $CaCO_3$ (no. 1), gave marked effects.

Penicillium roqueforti. This mould, grown for 17 days at 22° in medium purified by the Al_2O_3 method (no. 5), gave decreased sporulation and yields in the absence of each trace element. The final pH values were in the range 7·0–8·0, thus differing from *A. niger*, where pH values of 2·5 were not uncommon.

DISCUSSION

Many workers repeatedly recrystallize the ingredients of their media, and it is sometimes doubtful whether further purification by adsorbents or chelating agents achieves anything useful, but may even introduce contaminant trace metals or toxic substances. In the present work, methods of removing certain trace metals were tested, not with media made with recrystallized compounds, but with ingredients deliberately grossly contaminated with trace metals. In parenthesis, it may be noted that the A.R. products used frequently contained higher concentrations of Zn and Cu than did the corresponding C.P. or commercial grades.

It is not always clear in certain papers in the literature whether the 'complete' media used have been prepared by adding trace elements to purified or to unpurified media, thus making it difficult to decide whether the poorer growth sometimes recorded for purified medium was due to removal of micronutrients or to the introduction of toxic substances. In the present work, this uncertainty was eliminated by replacing optimum concentrations of micronutrients in media which had been treated to remove trace metals, and using the reconstituted medium as complete medium. Poor growth, or absence of growth due to toxic substances introduced into the medium, was sometimes found after treatment with H_2S or oxine. This is particularly important where chelating agents are used, since they are extremely difficult to remove completely from the medium, and their toxicity is influenced by the trace metal composition of the medium (Rubbo, Albert & Gibson, 1950;

Anderson & Swaby, 1951). In many media there is enough magnesium and phosphate to precipitate magnesium phosphate on autoclaving at neutral pH, and since this precipitate is able to remove traces of certain metals, the usefulness of an additional adsorbent may be in doubt. To obviate this criticism, the present authors used low concentrations of magnesium which, however, were sufficient for maximum growth. Sometimes all the salts used were reported to have been treated with a chelating agent except the $MgSO_4.7H_2O$ which, in our experience, is often grossly contaminated with heavy metals.

None of the methods tested for removing trace elements is entirely satisfactory for studying bacterial nutrition. Several species of bacteria capable of growth in the medium used for *A. niger* gave such small amounts of cells in complete medium that it is doubtful whether they would have exhausted the residual traces of micronutrients in purified media sufficiently to show decreased growth. Methods which use chelating agents are more laborious, may introduce toxic substances, and give no greater deficiencies than the Al_2O_3 method, which proved the most efficient of the adsorption methods for removing Fe, Zn, Cu and Mn. It is important to stress the need for swirling the flask to suspend the Al_2O_3 both before and after autoclaving. Filtration through acid-washed filter-paper is not as satisfactory as filtration through a fine sintered-glass filter connected by ground glass joints to a vacuum flask. There is no advantage in using a column of adsorbent. Slightly greater deficiency symptoms are achieved by repeating the Al_2O_3 treatment several times on one batch of medium. It is not known how adsorbents remove cations of trace metals, but the process may be analogous to cation exchange with complex silicates and synthetic resins, since for all these there is an optimum pH value for maximum effect and desorption can be induced by high concentrations of salts. Adsorbents like Al_2O_3, metal sulphides, and $CaCO_3$, which probably possess lower electrical charges per unit surface than do silicates and resins, are preferable to these last two adsorbents, since they do not appreciably affect the major element constitution of the medium.

The authors wish to acknowledge financial assistance from the Commonwealth Research Grant. They are also indebted to Dr E. G. Mulder and Dr F. C. Gerretsen for providing their strains of *A. niger*, and to Mr S. Woodward-Smith of the Department of Medical Artistry, University of Sydney, for the photographs.

REFERENCES

Anderson, B. I. & Swaby, R. J. (1951). Factors influencing the fungistatic action of 8-hydroxyquinoline (oxine) and its metal complexes. *Aust. J. sci. Res.* B, **4**, 275.

Bortels, H. (1927). Über die Bedeutung von Eisen, Zink und Kupfer für Mikroorganismen. *Biochem. Z.* **182**, 301.

Foster, J. W. (1949). *Chemical Activities of Fungi.* New York: Academic Press Inc.

Gerretsen, F. C. (1948). On the use of *Aspergillus niger* for the determination of plant nutrients in the soil. *Anal. Chim. Acta*, **2**, 782.

Mulder, E. G. (1948). The microbiological estimation of copper, magnesium and molybdenum in soil and plant material. *Anal. Chim. Acta*, **2**, 793.

NICHOLAS, D. J. D. (1950). Use of *Aspergillus niger* for determining magnesium, copper, zinc and molybdenum in soils. *J. Sci. Food Agric.* **1**, 339.

PIPER, C. S. (1944). *Soil and Plant Analysis.* Adelaide: University of Adelaide Press.

RUBBO, S. D., ALBERT, A. & GIBSON, M. I. (1950). The influence of chemical constitution on antibacterial activity. V. The antibacterial action of 8-hydroxyquinoline (oxine). *Brit. J. exp. Path.* **31**, 425.

STEINBERG, R. A. (1919). A study of some factors in the chemical stimulation of the growth of *Aspergillus niger*. *Amer. J. Bot.* **6**, 330.

STEINBERG, R. A. (1950). Trace element impurities in nutrient solutions for fungi. *Arch. Biochem.* **28**, 111.

WARING, W. S. & WERKMAN, C. H. (1942). Growth of bacteria in an iron-free medium. *Arch. Biochem.* **1**, 303.

EXPLANATION OF PLATES

PLATE 1

Appearance of *A. niger* strain M grown on defined medium purified with Al_2O_3 (method no. 5), showing characteristic changes brought about by each specific deficiency.

PLATE 2

Appearance of shaken cultures of *A. niger* strain M grown in defined medium purified with Al_2O_3 (method no. 5), showing characteristic changes brought about by each specific deficiency.

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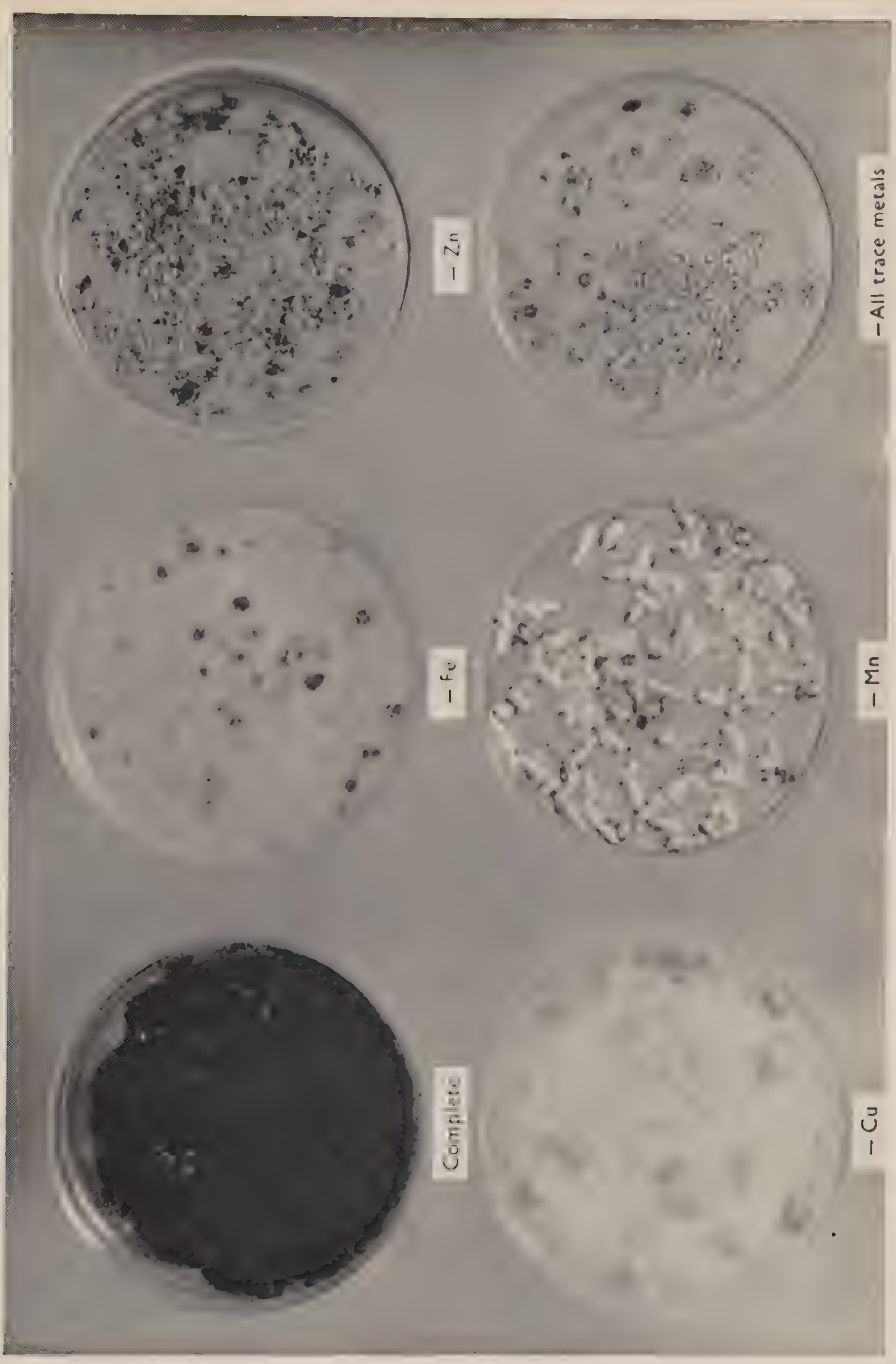
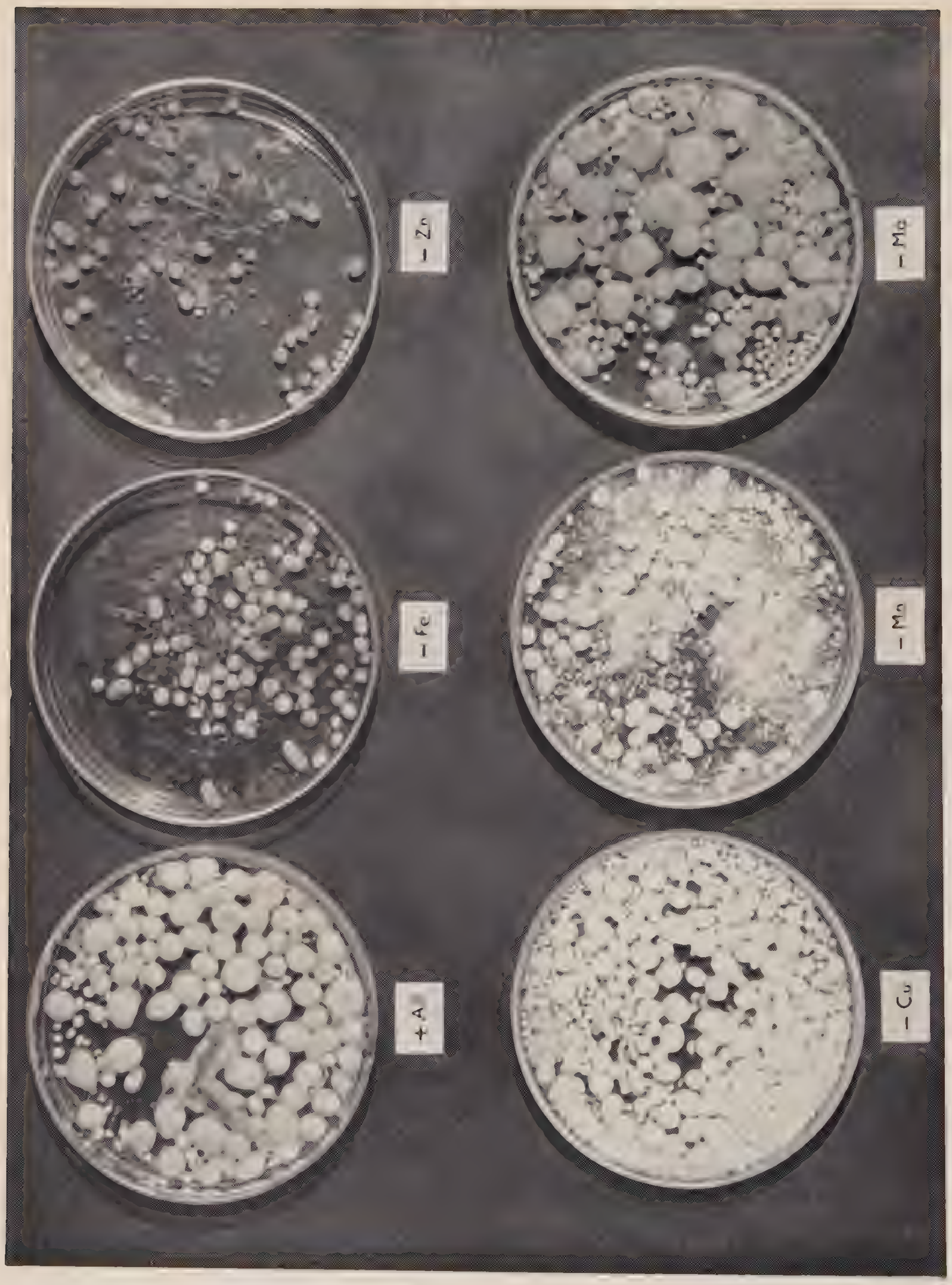

– Zn
– All trace metals
– Fe
– Mn
Complete
– Cu

Catherine Donald, Beverley I. Passey and R. J. Swaby—Removal of trace metals from media. Plate 1



Catherine Donald, Beverley I. Passey and R. J. Swaby Removal of trace metals from media. Plate 2

AJL, S. J. (1952). *J. gen. Microbiol.* 7, 221–232.

Factors affecting Oxidation of Acetate by *Bacterium coli*

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SUMMARY: Optimum conditions for acetate oxidation by acetate-adapted cells of *Bacterium coli* and experiments dealing with the inhibition of this oxidation are described. Quantitative relationships in isotope experiments between precursor and product implicate at least succinate, fumarate and malate in the *cyclic* mechanism of acetate oxidation by this organism. The involvement of additional compounds in the oxidation of acetic acid is not excluded by these experiments. However, compounds more oxidized than acetate, such as glycollate and glyoxylic acid, are shown not to be involved in the oxidation of the C_2-fatty acid.

Previous investigations (Slade & Werkman, 1943; Ajl, 1950; Ajl & Kamen, 1950; Swim & Krampitz, 1950; Barron, Ardeo & Hearon, 1950; Ajl & Kamen, 1951*a*; Dagley, Dawes & Morrison, 1951) have suggested that acetic acid may be oxidized by some bacteria to CO_2 and water via a dicarboxylic acid cycle initiated by a Thunberg-Knoop condensation. It was shown, for example (Ajl, 1950), that suspensions of *Bacterium coli* can be adapted to oxidize acetate rapidly. Such cells also oxidize succinate, fumarate, malate, oxaloacetate and pyruvate more readily than unadapted cells. However, the rate of oxidation of α-ketoglutarate or *cis*-aconitate is not increased, while citric acid is not attacked at all. Ajl & Kamen (1951*a*) found that when *Bact. coli* oxidized simultaneously both labelled acetate and unlabelled succinate, pyruvate, α-ketoglutarate, fumarate, malate and oxaloacetate, singly or in combination, then incorporation of isotopic carbon took place in all substrates with the exception of α-ketoglutarate. The isotopic distribution in the carriers and in the CO_2 evolved demonstrated that during acetate oxidation the methyl carbon of acetate was in equilibrium with the carbon of all the other substrates, except α-ketoglutarate. All substrates were metabolized, which appeared to exclude uncertainties in interpretation arising from differential permeability. There was a large endogenous reduction of the C_4-dicarboxylic acids tested with formation of succinate under aerobic conditions, even when oxidation was proceeding vigorously (Ajl & Kamen, 1951*a*). This endogenous reduction was considerably decreased under high oxygen tensions (Ajl, 1951*a*). Isotopic distribution in succinate and other dicarboxylic acids, as well as in pyruvate and evolved CO_2 suggested a cyclic mechanism. However, more quantitative data were required to show the nature of the cyclic mechanism involved; those studies form the main subject of the present paper.

In the present communication data on variation due to time in specific ^{14}C content of three intermediates during oxidation of labelled acetate are presented. The results are equally applicable whether a di- or tri-carboxylic acid cycle is operative. As far as we can ascertain, no such data have yet been presented in connexion with similar problems either in animals or micro-organisms. Further, results will be described which eliminate other possible routes of acetic acid oxidation by *Bact. coli*. In addition, certain optimum conditions will be described for the oxidation of acetate by this organism and experiments dealing with the inhibition of this oxidation will also be dealt with briefly.

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MATERIALS AND METHODS

Bact. coli (Iowa State E26) was grown for 48 hr., with constant aeration at approximately 30° in the usual medium (Ajl, 1950) containing sodium acetate as the sole carbon source. The handling of the cells and the chemical procedures were identical with those described (Ajl & Kamen, 1951*a*).

Respiratory gas exchanges were determined with the Warburg apparatus in the usual manner.

Radioassays were made, using a conventional end window G-M tube connected to a Tracerlab 'autoscaler'. Solutions were pipetted on recessed stainless steel disks after neutralization. Solids, such as cells, were mixed with ethanol and spread evenly on disks. The samples were dried under an infra-red lamp. Appropriate corrections for self-absorption and carbon content were made.

Experiments with acetate and possible products of its oxidation

Two types of experiment were performed. In procedure 1 a ^{14}C labelled intermediate was incubated in the presence of several unlabelled compounds to which the radioactive intermediate was thought to give rise. For example, radioactive succinate was incubated in the presence of unlabelled fumarate, malate, oxaloacetate, pyruvate and acetate. In like manner, radioactive fumarate was incubated in the presence of inactive malate, oxaloacetate, pyruvate and acetate, etc. Approximately uniformly ^{14}C labelled succinate was prepared by the method of Ajl & Kamen (1951*b*). Radioactive fumarate and malate were prepared in a similar manner, except that when labelled fumarate or malate was desired these, instead of succinate, were added as carriers during the oxidation of highly labelled acetic acid. In procedure 2 methyl-labelled acetate was incubated in the presence of relatively high concentrations of three intermediates (succinate, fumarate and malate) considered to be possible intermediates in the oxidative breakdown of the C_2-fatty acid.

Oxygen was passed constantly through the reaction mixture in 100 ml. test-tubes in order to diminish the reduction of C_4-dicarboxylic acids back to succinate (Ajl, 1951*a*). The CO_2 evolved was caught in alkali. The isolation procedures for succinate, fumarate and malate have been described by Ajl & Kamen (1951*a*), and the methods used for their quantitative estimation by Ajl (1951*a*). Oxaloacetate and pyruvate (but not α-ketoglutarate) were re-isolated chromatographically as their phenylhydrazones (LePage, 1950). Their identity was checked spectrophotometrically.

Experiments with glycollate, tartaric acid, glycolaldehyde and glyoxylate

When glycollic or tartaric acids were used as carriers, the procedures were as previously described (Ajl & Kamen, 1951*a*). Because of the instability of glyoxylic acid and glycolaldehyde, some modifications were introduced in re-isolating them from the reaction mixture. Instead of distilling off the residual acetate in steam after the addition of H_2SO_4, and removal of cells by centrifugation, the entire solution (including the acetate) was placed on filter-paper and partitioned between a mixture of *tert.*-amyl alcohol, formate and water (Lugg & Overell, 1948). Further, instead of removing the formic acid by autoclaving, the sheets were allowed to dry at room temperature for 18 hr. before spraying with bromcresol purple. Although it is not clear whether it is glyoxylic acid and glycolaldehyde, or the more oxidized forms of these compounds which ultimately separate out on paper, a definite band, with a constant R_f, is obtained when glyoxylic acid or glycolaldehyde is put on paper, either in pure form or when re-isolated after incubation with cells.

RESULTS

Phosphate requirement. The involvement of inorganic phosphate in the oxidation of a number of compounds including acetate is shown in Table 1. It was impossible to stop the oxidation of acetate completely by omitting phosphate. The question was raised whether the effect of phosphate might have been due either to the inability of the cells to carry out an initial phosphorylation of acetate or to a partial failure of the oxidation of some or all of the intermediates involved in the oxidation of acetic acid. (Phosphate is necessary for the optimum oxidation of all the presumed intermediates in acetate breakdown.) A different approach based on the following considerations was therefore adopted: if the initial step in acetate oxidation requires phosphate, then on incubating radioactive acetate in the presence of any intermediate, for example, succinate, with and without phosphate, the activity in the re-isolated carrier could be used as an index of phosphate requirement. This can be done only if the concentration of the carrier is so high

Table 1. *Involvement of phosphate in oxidation reactions*

Total volume of reactants 2·3 ml. Substrates were added in 100 μmol. quantities; 0·5 ml. of 0·2 M-phosphate buffer, pH 7·0 was added where indicated. Each flask contained 0·5 ml. of a 10% suspension of (12 times washed) *Bact. coli*. NaOH in centre well and distilled water to volume. Temp. 30°. Aerobic. Time of incubation, 90 min.

	Oxygen uptake (μl.)	
Substrate	With PO_4	Without PO_4*
Acetate	392	108
Succinate	390	266
Fumarate	310	210
Malate	380	200
Pyruvate	410	240

* The flasks without phosphate buffer contained veronal buffer.

that for all practical purposes it could be considered constant throughout the course of the experiment. The results of such an experiment (Table 2) show that phosphate is certainly required for the incorporation of acetate carbon into succinate. As with succinic acid oxidation (Hersey & Ajl, 1951) arsenate could not replace phosphate (Table 3) in acetate oxidation. It is to be noted in connexion with experiments dealing with the phosphate requirements that veronal buffer did not cause any inhibition of acetate oxidation because oxygen uptake was the same when phosphate buffer alone or when phosphate plus veronal buffers were used together in the same flasks.

Table 2. *Phosphate in acetate oxidation*

Each flask contained 20 μmol. of 2-^{14}C-acetate containing 10,000 counts/min.; 200 μmol. inactive succinate; 0·5 ml. of a 10 % suspension of (12 times washed) *Bact. coli* and 0·3 ml. of NaOH in centre well. 0·3 ml. of 0·2 M-phosphate or veronal buffer was added where indicated. Temp. 30°. Aerobic.

Additions to 2-^{14}C-acetate and carrier succinate	Activity in re-isolated succinate (counts/min.)
Cells	30
Cells plus phosphate buffer	1322
Cells plus veronal buffer	63

Table 3. *Arsenate in acetate oxidation*

Total volume of reactants 2·3 ml. Each flask contained 50 μmol. of sodium acetate; 0·5 ml. of a 10 % suspension of *Bact. coli*; 0·3 ml. NaOH in centre well. Phosphate or arsenate were added to respective cups (both were added at 0·2 M concentrations, pH 7·0, 0·5 ml.). Distilled water to volume. Time 120 min. Temp. 30°. Aerobic.

	Oxygen uptake (μl.)	
Substrate	Phosphate	Arsenate
Acetate	600	60

Inhibition by o-*dinitrophenol and sodium azide.* Some further evidence of the connexion between the phosphate requirement and the oxidative mechanisms concerned in acetate metabolism comes from experiments with *o*-dinitrophenol and sodium azide. Both of these compounds are known to uncouple oxidation from phosphorylation, e.g. in the presence of these reagents oxidation of substrate continues but phosphorylation is inhibited. At pH 6 (though not at pH 7·0) the oxidation of acetate is markedly inhibited (Table 4). This inhibition can perhaps be explained on the basis that some kind of an initial phosphorylating mechanism precedes the oxidation of acetate, a reaction presumably inhibited by dinitrophenol and sodium azide.

Effect of pH *value.* The oxidation of acetate is markedly affected by the pH value of the buffer used. The optimum pH value for acetate oxidation appears to be between 7·0 and 7·4 (Fig. 1). Similar results were obtained by Barron *et al.* (1950) with *Corynebacterium creatinovorans.*

Hydrogen transfer. The inhibition of acetate oxidation by sodium cyanide strongly suggests that electron transfer to molecular oxygen occurs through the cytochrome-cytochrome oxidase system (Table 5).

Table 4. *Inhibition of acetate oxidation by* o-*dinitrophenol and sodium azide*

Total volume of reactants 2·3 ml. Each flask contained 0·5 ml. 0·2 M-phosphate buffer at the pH indicated; 0·5 ml. of a 10% suspension *Bact. coli*; NaOH in centre well and distilled water to volume. 0·5 ml. of 1×10^{-3} M-*o*-dinitrophenol where indicated. Temp. 30°. Time 90 min. Aerobic. Exp. 2. Same conditions as Exp. 1 except that it was carried out at a different time with different cells. 0·5 ml. of 0·01 M-NaN_3.

Exp.	Substrate	Inhibitor	pH reaction medium	Oxygen uptake (µl.)	Inhibition (%)
1	Acetate	—	7·0	682	16
		Dinitrophenol	7·0	580	
		—	6·0	206	92
		Dinitrophenol	6·0	16	
2	Acetate	—	7·0	892	21
		NaN_3	7·0	702	
		—	6·0	468	95
		NaN_3	6·0	42	

Table 5. *Inhibition of acetate oxidation by* NaCN

Total volume of reactants 2·3 ml. Each flask contained 0·5 ml. of 0·2 M-phosphate buffer pH 7·0; 0·5 ml. of a 10% suspension of *Bact. coli*; NaOH in centre well and distilled water to volume. 0·5 ml. of 1×10^{-3} M-NaCN was added. Time 90 min. Temp 30°. Aerobic.

	Oxygen uptake (µl.)	
Substrate	With NaCN	Without NaCN
Acetate	2	700

Table 6. *Effect of keto fixatives on acetate oxidation*

Total volume of reactants 2·8 ml. Each flask contained 50 µml. of sodium acetate; 0·5 ml. of 0·2 M-phosphate buffer (pH 7·0); 0·5 ml. of a 10% suspension of *Bact. coli* and NaOH in centre well. Inhibitors were added to requisite cups. Temp 30°. Time 30 min. Aerobic.

Inhibitor	Conditions	Oxygen uptake (µl.)
—	pH 7·0	668
Bisulphite	pH 7·0; 0·005 M	5
$NH_2OH.HCl$	pH 7·0; 0·2 M	3
$NH_2OH.HCl$	pH 7·0; 0·002 M	174

The addition of keto fixatives to cell suspensions of *Bact. coli* inhibited the oxidation of acetate (Table 6); this suggests that some type of keto compound is involved in the oxidation.

Experiments with acetate and possible products of its oxidations

Previous work showed that upon incubating radioactive acetate in the presence of inactive C_4-dicarboxylic acids or pyruvate, all compounds become active (Ajl & Kamen, 1951*a*). In connexion with certain aspects of the present investigation it was necessary to show as unambiguously as possible that the

C_4 dicarboxylic acids and pyruvate can, among themselves, be interconverted. Accordingly, radioactive succinate was incubated in the presence of the other Szent-Györgyi C_4 acids, pyruvate and acetate, labelled fumarate in the presence of unlabelled malate, oxaloacetate, pyruvate and acetate, and labelled malate in the presence of unlabelled oxaloacetate, pyruvate and acetate. The results are shown in Table 7. Two observations deserve comment. First, it is clear that all the C_4-dicarboxylic acids are, during this normal aerobic metabolism, interconverted; secondly, both acetate and pyruvate become active in all cases.

Table 7. *Interconversion of* C_4*-dicarboxylic acids, pyruvate and acetate by* Bact. coli

Total volume of reactants 30 ml. Each flask contained 1 g. cells (wet weight) and 5 ml. of 0·2 M phosphate buffer pH 7·0. To appropriate flasks were added 10 ml. of radioactive 'starting material' and 100 μmol. of each carrier as indicated. The active mixture was incubated at room temperature with constant aeration (tank O_2) for 60 min.

			Activity in re-isolated carriers (counts/min.)				
Radioactive 'starting material'	Total activity (counts/min.)	Carriers	Fumarate	Malate	Oxalo-acetate	Pyruvate	Acetate
Succinate	57,520	Fumarate, malate, oxalo-acetate, pyruvate, acetate	6,400	4,700	5,500	4,600	3,200
Fumarate	30,600	Malate, oxaloacetate, pyruvate, acetate	—	5,220	4,300	2,600	3,000
Malate	20,100	Oxaloacetate, pyruvate, acetate	—	—	2,700	3,000	4,600

A method for the determination of an immediate precursor of a given compound has been presented by Zilversmit, Entenman & Fishler (1943). Using differential equations, they demonstrated that the following three criteria are the most useful in determining the precursor/product relationships: (1) the specific activity of the immediate precursor is greater than that of the compound arising from the precursor before the latter reaches its maximum specific activity; (2) after the compound reaches its maximum activity, the slope of the 'specific activity/time' curve of the compound is negative, and therefore the specific activity of the compound is greater than that of its precursors; (3) at the time when the compound has reached its maximum specific activity, the slope of its 'specific activity/time' curve is zero, and therefore the specific activity of the immediate precursor equals the specific activity of the compound at that time.

Fig. 2 clearly shows that all three criteria are satisfactorily met as far as succinate, fumarate and malate are concerned, when these are oxidized in the presence of labelled acetic acid. While the specific activity of fumarate is increasing, the specific activity of succinate is, as it should be, at all times greater than that of fumarate before the specific activity of the latter reaches a maximum. This satisfies condition 1. After fumarate reaches its maximum specific activity, the slope of the curve becomes negative, the specific activity relations are reversed, fumarate having a higher specific activity than succinate. This satisfies condition 2. At the maximum or near maximum specific activity of fumarate, the slope of its 'specific activity/time' curve is zero, and the specific activities of succinate and fumarate are equal, thus satisfying the third condition for the establishment of a precursor/product relation. Good correlation has also been obtained between fumarate and malate. It follows then that in normal metabolism of acetic acid, succinate, fumarate and malate are involved in the order indicated. This is true whether a di- or tri-carboxylic acid cycle operates in the oxidation of the C_2 acid.

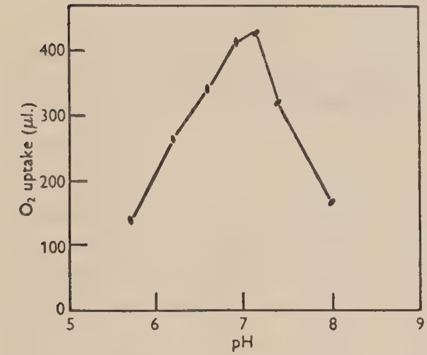


Fig. 1

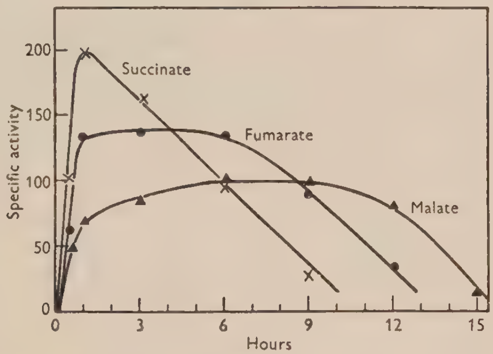


Fig. 2

Fig. 1. Effect of pH on acetate oxidation. Total volume 2·3 ml. Each flask contained 0·2 M phosphate buffer of desired pH; 0·5 ml. of a 10% suspension of *Bact. coli*; 50 μmol. of acetate and NaOH in centre well. Distilled water to volume. Temp 30°. Time 60 min. Aerobic. O_2-uptake in μl.

Fig. 2. Time-function relating specific activity of precursor to product during oxidation of 2-^{14}C-acetate. Each flask contained 150 μmol. of succinate, fumarate and malate; 97 μmol. of 2-^{14}C-acetate containing 109,552 counts/min.; 1 ml. of phosphate buffer (pH 7·0; 0·2 M); 1 ml. of a 20% suspension of acetate-adapted *Bact. coli*. Total volume 20 ml. The flasks were aerated with tank O_2 for the times indicated. Specific activity, counts/min./μmol. substrate.

Experiments with glycollate and glyoxylate

A possible mechanism of acetate oxidation is via glycollic and glyoxylic acids. Such an oxidation would presumably proceed as follows:

$$\underset{\text{Acetate}}{CH_3.COOH} \longrightarrow \underset{\text{Glycollate}}{CH_2OH.COOH} \longrightarrow \underset{\text{Glyoxylate}}{CHO.COOH}$$

This scheme of reaction has actually been suggested by Tolbert, Clagett & Burris (1949) to be involved in a C_2–C_2 condensation to a compound like dihydroxymaleic acid. On theoretical grounds only, we have considered such a mechanism unlikely (Ajl, 1951*a*). Experimental evidence against these compounds as intermediates in acetate oxidation will now be presented.

Manometric experiments. Fresh thoroughly washed 18 hr. old cells grown on acetate oxidize acetate and glycollate vigorously; glycolaldehyde and tartaric acid fairly rapidly; dihydroxymaleic acid to a small extent. However, when the same cells are aged, e.g. kept for a week or 10 days in the ice-box at 5°, the ability to oxidize acetate remains but the activity on all the other substrates almost completely disappears (Table 8). It appears, therefore, that although fresh acetate-grown cells do attack glycollate, glyoxylate, glycolaldehyde and tartaric acid, oxidation of acetate can continue when these intermediates cannot be attacked. If the oxygen uptake in 30 min. is taken as 100 for the old cells, the rate of oxidation of glycollic acid is 3, of glyoxylic acid 10, and of tartaric acid 7. Similar results were obtained by Barron *et al.* (1950) with *C. creatinovorans.*

Table 8. *Metabolism of possible intermediates of acetate oxidation by acetate-adapted* Bact. coli

Total volume of reactants 2·3 ml. Each flask contained 50 μmol. of substrate; 0·5 ml. of 0·2 M-phosphate buffer pH 7·0; 0·5 ml. of a 10% suspension of cells; NaOH in the centre well and distilled water to volume. Aerobic. Time 30 min.

	Oxygen uptake (μl.)						
Enzyme	Acetate	Glycollate	Glyoxylate	Glycolaldehyde	Tartaric acid	Dihydroxymaleic acid	Acetaldehyde
Aged cells*	169	8	18	5	12	7	12
Fresh cells*	323	296	173	100	101	22	43

* Both kinds of cells exhibited negligible endogenous activity.

Evidence of a more direct nature was obtained by the use of labelled acetate. Two preliminary experiments were performed, in each of which labelled acetate was partially oxidized in the presence of either a mixture of unlabelled succinate, glycollate and tartaric acid, or a mixture of succinate and glyoxylate. Results are exhibited in Table 9; without exception, only succinate became radioactive.

Table 9. *Distribution of acetate carbon in possible intermediates of acetate oxidation during the aerobic metabolism of* $2\text{-}^{14}\text{C}$ *acetic acid*

Total volume of reactants 11 ml. Each flask contained 100 μmol. of respective carriers; 1 ml. of 0·2-M; phosphate buffer; 1 ml. of freshly harvested acetate-grown *Bact. coli*; 60 μmol. of $2\text{-}^{14}\text{C}$-acetate (30,000 counts/min.); NaOH in centre well and distilled water to volume. Aerobic. Time of incubation, 180 min.

Additions to $2\text{-}^{14}\text{C}$- acetate	Total activity in re-isolated carrier (counts/min.)
Succinate	5000
Glycollate	200
Tartaric acid	90
Succinate	6000
Glyoxylate	74

In these experiments no data were obtained indicating whether glycollate or glyoxylate were actually metabolized during the course of oxidation of $2\text{-}^{14}\text{C}$-acetate, consequently uncertainties due to permeability of those acids could not be adequately excluded. In another series of experiments the utilization of glycollate and glyoxylate was confirmed by oxygen uptake measurements. There was a considerable O_2-uptake with acetate alone but this was nearly doubled when glycollate, glyoxylate or tartaric acid were added (Table 10). It is very likely, therefore, that these acids were metabolized. Neither glyoxylate nor tartaric acid contained any radioactivity; the possible significance of the 156 counts/min. found in glycollate will be considered later.

Table 10. O_2-*uptake and radioactivity data obtained during the breakdown of* 2-^{14}C-*acetate in the presence and absence of carriers*

Total volume of reactants 3 ml. Each flask contained 60 μmol. of 2-^{14}C-acetate (30,000 counts/min.); 0·5 ml. of 0·2-M phosphate buffer pH 7·0; 0·5 ml. of a 10% suspension of 3-day-old acetate-adapted cells of *Bact. coli*; NaOH in centre well. Carriers were added in 50 μmol. quantities where indicated. Distilled water to volume. Aerobic. Time 90 min.

	O_2-uptake		Distribution of activity in re-isolated carriers (counts/min.)					
Additions to 2-^{14}C-acetate	With acetate (μl.)	Without acetate (μl.)	Succinate	Fumarate*	Malate*	Glycolate	Glyoxylate	Tartaric acid
—	351	75	178*	—	—	—	—	—
Succinate	772	739	5120	640*	896*	—	—	—
Glycollate	504	278	512*	—	—	156	—	—
Glyoxylate	515	322	514*	—	—	—	Bkgd.	—
Tartaric acid	607	496	640*	—	—	—	—	Bkgd.

* Formed during the course of incubation period. Bkgd. = background.

Even from the O_2-uptake data shown in Table 10, there is no absolute assurance that both acetate and the intermediate carriers were oxidized. A better criterion would be to trap the CO_2 and, if starting with methyl-labelled acetate, the CO_2 became active and the intermediate carriers did not, even though the latter were actively metabolized as shown by oxidative data, then it could be assumed with somewhat more certainty that they were not involved in the main metabolic pathway of acetic acid oxidation. With this in mind, an experiment was performed and the data obtained are exhibited in Table 11. The results clearly show that, although the intermediates as well as the acetate were being actively oxidized, glycollate, glyoxylate or glycolaldehyde did not become appreciably radioactive. The high radioactivity of the CO_2 indicates vigorous acetate oxidation by a cyclic process since the labelled carbon was originally in the methyl group.

DISCUSSION

The cyclic mechanism of acetate oxidation appears now to be rather well established. It had been shown previously by Ajl & Kamen (1951*a*) that the oxidation of 2-^{14}C-acetate resulted in nearly symmetrical distribution of radioactivity in succinate; in addition, the radioactivity of the CO_2 evolved was nearly equal to the ^{14}C content of succinyl carboxyl. The actual order of the reactions in the cycling process are further indicated by the ‘time/specific activity data' reported in the present paper. The data appear to fit the theoretical criteria that have been set up for precursor/product relationships.

Theoretically acetate could be oxidized via glycollic and glyoxylic acids. Such a mechanism would be attractive from a chemical standpoint because it involves the well-known aldol type of condensation. However, the experiments presented appear to eliminate the possibility of a glyoxylic condensation mechanism. Glycollate, glyoxylate or glycolaldehyde are all ineffective in trapping acetate carbon, under conditions in which acetate carbon is oxidized and trapped in succinate, fumarate and malate. From the data in Table 9, for instance, only $<0{\cdot}5$ μmol. of acetate carbon was trapped in glycollate and approximately 0·1 μmol. in glyoxylate, whereas under the same conditions succinate trapped between 10 and 12 μmol. of acetate carbon.

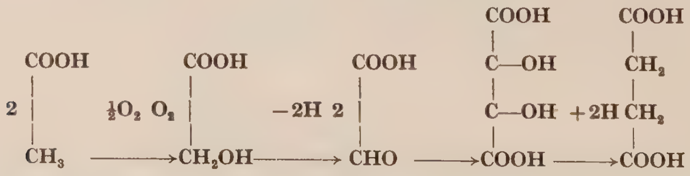
Table 11. *O_2-uptake and CO_2 data obtained during the oxidation of 2-^{14}C-acetate in the presence and absence of carriers*

Each flask contained 50 μmol. of 2-C^{14}-acetate (5000 counts/min.); 0·5 ml. 0·2 M-phosphate buffer pH 7·0; 0·5 ml. of a 10 % suspension of acetate-adapted *Bact. coli*, and 50 μmol. of carrier substance where added; NaOH in centre well. Total volume 3 ml. Distilled water to volume. Aerobic. Time 90 minutes.

Additions to 2-^{14}C-acetate	O_2-uptake with acetate (μl.)	Distribution of activity (counts/min.)						
		CO_2	Succinate	Fumarate	Glycolate	Glyoxylate	Glycolaldehyde	Cells
—	316	2268	—	—	—	—	—	1443
Succinate	771	640	702	298*	—	—	—	725
Fumarate	644	658	565*	887	—	—	—	806
Glycollate	468	1881	—	—	130	—	—	1366
Glyoxylate	464	1976	—	—	—	51	—	1223
Glycolaldehyde	468	2426	—	—	—	—	273	1274

* Formed during the course of incubation period.

It should be stressed that some radioactivity was always found in glycollate, as well as in glyoxylic acid. However, the counts could not be considered of significance as far as acetate oxidation is concerned because of the precursor-product relationships previously discussed. A possible way in which these acids could participate in acetate oxidation would be as follows:



$$2\,\begin{matrix}\mathrm{COOH}\\|\\\mathrm{CH_3}\end{matrix}\xrightarrow{\frac{1}{2}O_2\ O_2}\begin{matrix}\mathrm{COOH}\\|\\\mathrm{CH_2OH}\end{matrix}\xrightarrow{-2H}2\,\begin{matrix}\mathrm{COOH}\\|\\\mathrm{CHO}\end{matrix}\longrightarrow\begin{matrix}\mathrm{COOH}\\|\\\mathrm{C{-}OH}\\|\\\mathrm{C{-}OH}\\|\\\mathrm{COOH}\end{matrix}\xrightarrow{+2H}\begin{matrix}\mathrm{COOH}\\|\\\mathrm{CH_2}\\|\\\mathrm{CH_2}\\|\\\mathrm{COOH}\end{matrix}$$

This scheme presupposes that both glycollic and glyoxylic acids are precursors of succinate. Therefore, at any given time, if the concentration of all of these acids is approximately the same, the activity should be greater in the monocarboxylic acids than in the succinate; this was never the case.

The situation, however, appears to be somewhat more complex than the above data suggest. We have shown that cells freshly harvested from acetate medium and thoroughly washed, will oxidize glycollate, glyoxylate, glycolaldehyde and tartaric acid. Isotopic data show that these acids are not involved in acetate oxidation. Further, similarly treated cells from glucose medium will not attack either these acids or acetate appreciably (see Table 12). A full interpretation of these data is beyond the scope of this work. However, in view of the isotopic data, the latter results can best be interpreted as a non-specific, adaptive response of acetate-grown cells to glycolate and glyoxylate.

Table 12. *Oxidation of glycollate, glyoxylate, tartaric acid, dihydroxymaleic acid, glycolaldehyde, acetate and succinate by glucose-grown* Bact. coli

Total volume of reactants 2·3 ml. Each flask contained 50 μmol. of substrate; 0·5 ml. of 0·2 M-phosphate buffer pH 7·0; 0·5 ml. of a 10 % suspension of glucose-grown *Bact. coli*; NaOH in centre well and distilled water to volume. Aerobic. Time 30 min.

Oxygen uptake (μl.)						
Acetate	Glycolate	Glyoxylate	Glycol-aldehyde	Dihydroxy-maleic acid	Tartaric acid	Succinate
40	8	11	−7	−8	0	140

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REFERENCES

AJL, S. J. (1950). Acetic acid oxidation by *Escherichia coli* and *Aerobacter aerogenes*. *J. Bact.* **59**, 499.

AJL, S. J. (1951*a*). Studies on the mechanism of acetate oxidation by bacteria. V. Evidence for the participation of fumarate, malate, and oxalacetate in the oxidation of acetic acid by *Escherichia coli*. *J. gen. Physiol.* **34**, 785.

AJL, S. J. (1951*b*). Terminal respiratory mechanisms in microorganisms. *Bact. Rev.* **15**, 21.

AJL, S. J. & KAMEN, M. D. (1950). Studies on the mechanism of acetate-oxidation by bacteria. *Fed. Proc.* **9**, 143.

AJL, S. J. & KAMEN, M. D. (1951*a*). Studies on the mechanism of acetate-oxidation by *Escherichia coli*. *J. biol. Chem.* **189**, 845.

AJL, S. J. & KAMEN, M. D. (1951*b*). A simple and rapid biosynthesis of isotopically labelled succinic acid. *J. Amer. chem. Soc.* **75**, 2349.

BARRON, E. S. G., ARDEO, M. S. & HEARON, J. (1950). The mechanism of acetate oxidation by *Corynebacterium creatinovorans*. *Arch. Biochem.* **29**, 130.

DAGLEY, S., DAWES, E. A. & MORRISON, G. S. (1951). The kinetics of pyruvate production by *Aerobacter aerogenes*. *J. gen. Microbiol.* **5**, 508.

HERSEY, D. F. & AJL, S. J. (1951). Phosphorylation due to the oxidation of succinic acid by cell-free extracts of *Escherichia coli*. *J. gen. Physiol.* **34**, 295.

LEPAGE, G. A. (1950). Measurements of keto acids in normal and neoplastic rat tissue. *Cancer Res.* **10**, 393.

LUGG, J. W. H. & OVERELL, B. T. (1948). One and two-dimensional partition chromatographic separations of organic acids on an inert sheet support. *Aust. J. sci. Res.* A, **1**, 98.

Slade, H. D. & Werkman, C. H. (1943). Assimilation of acetic and succinic acids containing heavy carbon by *Aerobacter indologenes*. *Arch. Biochem.* **2**, 97.

Swim, H. E. & Krampitz, L. O. (1950). Evidence for the condensation of acetic acid to succinic acid in *Escherichia coli*. *Bact. Proc.* no. 125.

Tolbert, N. E., Clagett, C. O. & Burris, R. H. (1949). Products of the oxidation of glycolic acid and lactic acid by enzymes from tobacco leaves. *J. biol. Chem.* **181**, 905.

Zilversmit, D. B., Entenman, C. & Fishler, M. C. (1943). On the calculation of 'turnover time' and 'turnover rate' from experiments involving the use of labeling agents. *J. gen. Physiol.* **26**, 325.

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Bissett, K. A. (1952). *J. gen. Microbiol.* 7, 233–242.

Complete and Reduced Life Cycles in *Rhizobium*

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SUMMARY: Strains of *Rhizobium* from a wide variety of host plants were found to possess either a complete or a reduced life cycle. In the former, which was characteristic of strains from certain wild legumes and garden flowers, the bacteroids within the nodule were branched and septate; the free-living stages included small Bacterium-like forms which were responsible for infection of the host plant, and large, occasionally Gram-positive forms resembling *Bacillus*, which produced both specialized, coccoid swarmers and resistant endospores. The reduced cycle was commonly found in *Rhizobium* from cultivated field crops. The bacteroids were single cells of irregular outline; the free-living stages were reduced to the Bacterium-like stage alone, sometimes so actively motile as to perform the function of swarmers. This condition is regarded as degenerate and due to a parasitic habit. The mode of formation of the endospores, which differs in detail from that of *Bacillus*, is described. The morphology of various stages in the life cycle and a slight tendency to Gram-positivity render it apparent that *Rhizobium* is a specialized genus of Bacillaceae, probably related to the plant parasitic *B. polymyxa* group, with which it also has biochemical affinities.

Although *Rhizobium* has been much studied, attention has been almost exclusively diverted towards the strains or species which exist in symbiosis with important field crops. In a recent review Allen & Allen (1951) pointed out that many strains from less familiar legumes do not accord so closely with the classical descriptions, and Bisset & Hale (1951) reported that true swarmers are more readily produced by *Rhizobium* from lupins, sweet-peas, etc., than from cultivated peas and clovers, in which the function of swarmers is performed by small, motile bacilli, produced by normal cell division. In the present work further differences between the life cycle of these different types of *Rhizobium* are indicated.

The problem of the existence of endospores in *Rhizobium* was at one time the subject of considerable controversy, but it has been almost entirely forgotten of recent years (Lewis, 1938). The present attitude is well represented by Nutman (1952), who expressed surprise at the powers of survival of lucerne *Rhizobium*, ‘since *Rhizobium* does not form spores’. It is my experience that precisely this strain of *Rhizobium* forms spores very readily, which serves to explain its resistance. Here, and in a previous paper (Bisset, 1952*a*), evidence is presented to show that some, but not all, strains produce resistant endospores, and have, at certain stages of their life cycle, morphological characters which suggest a relationship with the true sporing bacilli.

METHODS

Thirty-four strains of *Rhizobium* were examined. Twenty-nine were newly isolated from the root nodules of pea, bean, everlasting sweet-pea, wild vetch, lupin, broom, tree-lupin and various species of clover. Five were laboratory cultures, of which all but one were of classical morphology.

Initial cultures were made by sterilizing nodules externally with mercuric chloride, crushing between sterile slides, and inoculating upon nutrient agar containing 0·3% Yeastrel. Subcultures were made upon this medium, or, for the production of 'bacteroids' such as appear in the root nodules, in a simple infusion of sweet-pea foliage. Strains which produced variants of unusual appearance were repeatedly plated for purity.

Preliminary observations

Because the evidence and conclusions presented in this paper are contrary to long-established belief upon the nature and morphology of *Rhizobium*, I think it best to describe the observations from which they arose, in the order and manner in which they occurred.

A large number of strains of *Rhizobium* was isolated in this laboratory in the course of an investigation upon the production of swarmers (Bisset & Hale, 1951). While this work was in progress it was observed that the morphology and cultural characters of many isolates were not in accordance with previous descriptions of the genus. Many were Gram-positive, especially in very young cultures. The degree of Gram-positivity varied, and most frequently consisted of little more than the appearance of a few granules, which disappeared as the culture aged. Some, however, were so strongly Gram-positive, and so closely resembled ordinary sporing bacilli that they were repeatedly discarded as contaminants until the failure to isolate any other bacteria from the same sources, together with the discovery of similar, Bacillus-like variants in stock cultures of unimpeachable authenticity and purity, led to their further investigation. It was then discovered that by every criterion of life cycle and plant symbiosis they were indeed *Rhizobium*. It was also apparent that, despite their resemblance to *Bacillus*, they had many characters quite unlike those of any known member of the genus; and that although they produced endospores capable of surviving boiling, these were formed in a manner which differed in detail from the cytological process found normally in *Bacillus* and *Clostridium*.

By the selection of heat-resistant strains, similar, partially Gram-positive, sporogenous strains were also obtained from isolates which were quite typical in appearance. These almost invariably lost their Gram-positivity and Bacillus-like morphology as the cultures aged, but in addition, reverse mutations to a typical form occurred in strongly Gram-positive sporogenous strains, giving rise once more to a strain which was Gram-negative and of characteristic *Rhizobium* morphology in all stages of culture.

The biochemical characters of the various strains were exceedingly variable, and frequently quite unlike the accepted reactions for *Rhizobium*, which is supposed to be incapable of producing detectable fermentation of carbohydrates in peptone water cultures. The reactions were equally unlike those of any species of *Bacillus* which might be expected to appear as accidental contaminants, in that both acid and gas were produced. Cultural characters are described in a later section; it suffices to record here that whereas certain strains, both Bacillus-like and typical, did in fact fail to ferment any of the carbohydrates tested, others produced acid and gas, very slowly, in a varied range of substrates. The biochemical reactions of typical and sporogenous variants from any one strain were always identical, whereas those from different sources were very seldom alike. In sweet-pea infusion and similar dilute media of acid reaction, branched bacteroids, typical of *Rhizobium*, were produced by strains of all types. Species of *Bacillus* did not produce these appearances. Thus all available evidence pointed to the identity of the Bacillus-like and typical *Rhizobium* strains.

In some strains, including the majority of those obtained from cultivated crops such as clover and peas, the small typical forms alone were found. Spores and true swarmers were both very rare. This is a highly significant finding, as it is exactly these types of *Rhizobium* which have been most fully studied in the past, and upon which the accepted descriptions are based. Strains of atypical morphology and behaviour were commonest in the nodules of wild legumes or garden flowers, which in the past appear to have been investigated very rarely. It is also highly probable, from my experiences in this work and in examination of practical exercises performed by students, that sporogenous strains have many times been isolated and discarded in the erroneous belief that they were contaminants. There is more than one hint of this in the literature (cf. the review by Allen & Allen, 1951), and occasional reports of Gram-positive variants verified to be *Rhizobium* (Kleczkowska, 1950).

Sporogenous strains of Bacillus-like morphology, and with a greater or less tendency to Gram-positivity, were isolated from lupin, everlasting sweet-pea, tree-lupin and broom, and from a stock culture derived from lucerne. The latter will be discussed separately. Strains which were morphologically typical, small and Gram-negative, but which produced visible spores within the cell, as observed by routine spore stains, and which survived boiling, were regularly isolated from vetch, and occasionally from clover.

Morphology

The cytology of endospore formation in *Rhizobium* is described in a later section. The large, 'barred' cells, from which swarmers were liberated, have frequently been described, and an account has been given of them elsewhere (Bisset & Hale, 1951). These were never Gram-positive, but although considerably larger in size they nevertheless strongly resembled *Bacillus* and the vegetative cells of the Bacillus-like variants of *Rhizobium* in the details of their cytology. The transverse bars, so often described in the past, are in fact cross-walls closely resembling those of multicellular species of *Bacillus* (Bisset, 1950, 1952*b*), but combined with a thick layer of basophilic material which thus lines the cells of which the bacillus is composed and is presumably concerned with the formation of the swarmers (Bisset & Hale, 1951). These cross-walls were absent from *Rhizobium* of typical morphology, the cytological structure of which resembled that of *Bacterium* (Bisset, 1950, 1952*b*), but were exceptionally well-marked in the sporogenous strains, whether strongly or weakly Gram-positive, or completely Gram-negative (Pl. 1, fig. 1). Paired nucleoids were seen in each of the four, six or more cells into which the bacillus was thus divided, exactly as in rough forms of *Bacillus* (Pl. 1, figs. 2, 3). Vegetative cells had a small number of peritrichous flagella; swarmers, one or two polar flagella (Bisset & Hale, 1951).

The morphology of the bacteroids in plant nodules or in sweet-pea infusion was distinctly different in strains which freely produced true swarmers and endospores from septate bacilli, from that of those strains in which typical forms alone were found. In the former the bacteroids also were large and septate and branched freely (Fig. 1A); in the latter they were unicellular or composed of one large, irregularly shaped cell, with a very small branch (Fig. 1D). Strains of intermediate characters, such as those from vetch, which produced endospores but not swarmers from typical, small, Gram-negative bacilli, were intermediate in this respect also and formed bacteroids with three or four cells and moderately frequent branches (Fig. 1B, C). Because of the amount of slime and plant debris in preparations of bacteroids good photomicrographs were difficult to make, and they have been illustrated only by drawings.

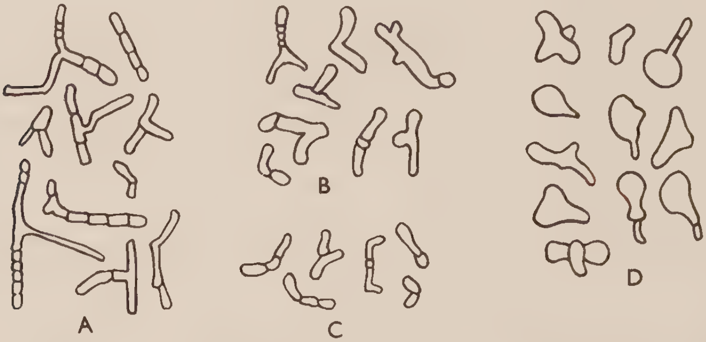


Fig. 1. Drawings of tannic-acid-violet preparations of bacteroids from root-nodules of various plants. A: from lupin; branched, multicellular type. B: from vetch; intermediate type. C: from cultivated bean; intermediate type. D: From cultivated pea; irregular, mainly unicellular, degenerate type.

Even the most Bacillus-like strains were immediately transformed into bacteroid morphology when subcultured in sweet-pea infusion. Their appearance in the nodule and in the culture was always the same for any one strain of *Rhizobium* and characteristic of the types described above. Cultures of a variety of species of *Bacillus* were grown in this medium but either retained their original morphology or failed to grow. These included *B. subtilis*, *B. megaterium*, and several strains each of *B. polymyxa*, *B. cereus* and *B. mycoides*. This provides additional proof that the sporogenous strains of *Rhizobium* are not contaminants.

Plant inoculation

In order still further to confirm the identity of the sporogenous strains plant inoculation experiments were performed. Cultures of an isolate from lupin were heated for 10 min. at 100°. Subcultures were made, from which suspensions were inoculated on lupin seeds which had previously been sterilized externally with 1/1000 mercuric chloride, and washed in sterile water. Twelve seeds were sown in autoclaved soil and watered with autoclaved water. Twelve control seeds were similarly treated but were not inoculated. When the seedlings were about 3 in. high the roots were washed and examined. The test seedlings had numerous well-formed nodules on the roots; the control seedlings had none. From the nodules of the test seedlings a strain of *Rhizobium* was isolated which again survived 10 min. at 100°, and had the same biochemical characters (see below) as the strain with which they had been inoculated. The recovered strain, at first, although sporogenous was Gram-negative and typical in appearance, like those recorded above from vetch and clover. It also resembled vetch strains in its tendency to produce transient orange pigmentation of the colony after heat-testing, a phenomenon discussed in the section on culture. This experiment proves that *Rhizobium* may pass through a heat-resistant stage while retaining the power of entering into symbiosis with the host plant.

Parallel experiments were made with lupin seeds sown upon nutrient salt agar slopes, in which the early stages of seedling growth and infection were observed. The original inoculum consisted entirely of Gram-positive, Bacillus-like forms, but after contact with the roots typical forms became more common, and these alone were found in crushed preparations of the roots.

A similar effect was observed in an experiment made with a strain of *Rhizobium* derived from a stock culture from lucerne. The original heat-resistance test produced a Bacillus-like strain in which, at first, no Gram-negative forms could be seen. After several weeks it again dissociated, giving rise to typical Gram-negative strains which did not survive boiling and which had completely changed their antigenic structure (antisera against the original strain were available for agglutination tests). The sporogenous strain from which they were derived was also antigenically different from the original and was not infective for lucerne in that condition. The newly dissociated typical variants, however, were fully infective for lucerne, thus confirming that normally infective *Rhizobium* may pass through a heat-resistant phase.

In a further experiment inoculation of lucerne seedlings, grown on agar slopes, with suspensions which had been boiled for 5 min. immediately before inoculation, produced poor, atypical nodules on the side-roots, from which a very slow-growing strain of *Rhizobium* was recovered. All these experiments appear to indicate that infection of the host plant is best achieved by bacteria of the typical Gram-negative, Bacterium-like morphology, and that reversion to this form may occur with varying readiness, either spontaneously or when sporogenous strains are brought into contact with susceptible plants. Infection by means of swarmers, which has been the subject of much comment by previous writers, was not specifically studied. It is, however, by no means the only mode of infection in *Rhizobium*.

Heat resistance

The character of heat resistance was very variable in the *Rhizobium* strains examined. As has been explained, some variants formed spores freely, some rarely, some never. The resistance of the spores themselves was also very variable, as was the after-effect of boiling on those strains which survived it. Some grew very slowly on first subculture after boiling, taking as long as 8 weeks or more to produce recognizable colonies. The colonies of isolates from vetch and lucerne, which normally were grey in colour, were bright orange on first subculture after boiling, but reverted to normal colour after two or three further subcultures. This also occurred, as described above, in the case of the lupin strain, re-isolated from experimental plant nodules. No explanation of this phenomenon can be offered.

The most resistant strains were those from lupin, vetch and everlasting sweet-pea, which survived boiling for 10 min. The standard test which eventually was used as a criterion of heat resistance consisted in heating a broth culture in a test-tube for 30 min. in a water-bath at 80°. At the end of this period the temperature was raised to 100° and maintained there for 5 min.

The variant from the stock culture of lucerne *Rhizobium* with which some of the plant inoculation experiments were performed was a source of much confusion because of its unpredictable heat resistance. It was the only stock culture from which resistant variants were isolated, and had been maintained in the laboratory for more than 20 years in artificial culture. The subcultures from the first heat test were discarded after showing no signs of growth in more than 2 weeks. A second test was made and in this instance the subcultures were left by chance for approximately 5 weeks before examination. On this occasion they were found to have grown. The sporogenous variant which arose survived boiling and typical variants from it were infective for lucerne, but the original strain invariably took an excessively long time to recover from boiling, which more often than not it failed to survive. Subcultures which did survive were among those which at first produced orange-pigmented growth, but which rapidly lost this character on further subculture.

Heat-resistance tests performed on this stock culture, at the institute from which it was obtained, consistently failed to produce resistant variants, although these were obtained in this laboratory on several occasions. The eccentric behaviour of this stock culture may be explicable by the long period since its first isolation, whereas the other sporogenous strains were all freshly isolated.

Cultural characters

In culture *Rhizobium* characteristically produces a voluminous pale-grey mucoid growth. Some of our cultures showed this appearance at all stages; in others the growth at first isolation was flat and granular, but changed after 2 or 3 days to the typical mucoid appearance. Granular colonies sometimes became surrounded with mucoid colonies, apparently derived from swarmers. Orange-pigmented colonies were occasionally formed by heat-resistant strains on first subcultures after boiling. Others grew very slowly after boiling.

Although the standard descriptions suggest that *Rhizobium* is incapable of producing recognizable fermentation of carbohydrates in peptone water, it is my experience that strains vary very much in this respect, although invariably slow in their reactions. Some, in fact, fermented nothing, but many produced acid and gas, under aerobic conditions, so that the fluid in the Durham tube remained unaltered long after the remainder had changed. Usually only a small range of substrates was affected. Acid reactions appeared in 2 or 3 days at 20°, but gas began to collect only after a week or more. As there is little or no correlation between the results given by different isolates and their morphological and other characters, and as these biochemical characters were extraordinarily variable, little purpose would be served by tabulating a large number. One important point, however, is that despite this variability, morphologically typical and atypical strains from the same isolate were always alike, e.g. the original and re-isolated strains from the lupin inoculation experiment were alike in fermenting only glucose; the sporogenous non-infective, the asporogenous infective, and the original, antigenically distinct, stock cultures from lucerne all failed to ferment any carbohydrate. Isolates from broom and lupin fermented only glucose; those from sweet-pea fermented glucose, sucrose and maltose; one strain from clover fermented glucose, lactose, mannitol, sucrose, rhamnose, aesculin, xylose, arabinose, maltose and salicin, but not dulcitol or inositol.

I am unable to account for the discrepancy between these results and the standard descriptions of the genus. These tests were made with strains which had been repeatedly plated for purity. Whereas the power to ferment carbohydrates was by no means confined to the morphologically atypical strains, but was especially remarkable in the typical strains from clover, nevertheless the five stock cultures were, without exception, completely or almost completely devoid of fermentative ability. It may thus be that this organism rapidly loses this power in artificial culture, and it is this which has been responsible for the existing descriptions.

The production of gas in fermentation, or total failure to ferment carbohydrates, are both very unusual characters in sporing bacilli. This decreases still further the possibility that the sporogenous strains were accidental contaminants. Gas production, however, is found in *B. polymyxa*, which also has a plant dwelling habit and is especially liable to the production of Gram-negative asporogenous variants. This possible relationship is referred to in the Discussion, but it is perhaps well to emphasize that the sporogenous *Rhizobium* strains are not identical with *B. polymyxa*, which ferments a standard series of substrates, does not form swarmers or bacteroids, and produces its spores in a cytologically distinct fashion.

Endospore formation

In those strains which exhibited the complete life cycle, spore-like resting cells and tiny coccal swarmers were frequently found to be formed in the same culture. The mother cells were always the large multicellular Bacillus-like stages (Pl. 1, figs. 1–3). In typical members of the genus *Bacillus* a single spore is formed, as a rule, in a four-celled bacillus. In the sporogenous strains of *Rhizobium*, however, it is not unusual for each cell to produce a spore. These remain enclosed within the cell wall of the sporangium until released by its rupture, and thus appear in long chains (Pl. 1, fig. 4–6). Apart from these points, the cytology of spore formation is similar to that seen in other sporogenous genera. A rod-shaped fusion-nucleus is formed (Pl. 1, fig. 7), and maturation is preceded by a reduction process, in which one nuclear element is rejected (Pl. 1, figs. 8, 9). After release from the sporangium, the spores germinate by the rupture and rejection of the spore coat (Pl. 1, fig. 10).

DISCUSSION

Strains of *Rhizobium* capable of surviving boiling by virtue of the production of resistant resting-cells closely resembling endospores, have been isolated from root-nodules in leguminous plants. Some, but not all, of these passed through a phase in which they resembled an ordinary Gram-positive sporing *Bacillus*, but differed from most of these in many respects, notably in their metabolism and in the mode of formation of the spore. They also possessed, even in this atypical phase, the typical *Rhizobium* characters of producing swarmers from barred cells and bacteroids in appropriate culture media which had the distinctive appearance of the bacteroids in the root-nodules from which they were isolated. True *Bacillus* species did none of these things. The identity of the Bacillus-like strains with *Rhizobium* is further proved by their ability after having survived boiling to infect the appropriate species of host plant (in which respect *Rhizobium* is highly specific) and to produce nodules in the roots. As a rule infection occurred only through the agency of variants of typical morphology; these were derived from atypical strains, either spontaneously or by the influence of contact with seedling plants. The identity of the typical and atypical variants was again confirmed by the fact that, despite the exceedingly variable biochemical reactions within the genus, those of variants from the same isolate were always alike. The classical description of the genus, as incapable of fermenting carbohydrates, is true of some strains, and especially of stock cultures, but it is not confirmed in the majority, irrespective of their morphological appearance. Some produced acid and gas in a small range, others in a much wider range of substrates, but all were very slow to do so.

Hints and suggestions in the literature (cf. Allen & Allen, 1951) make it apparent that such peculiarities in the morphology and cultural characters of *Rhizobium*, especially when derived from unusual host plants, are by no means unknown to workers in this field, but little beyond these hints has so far been ventured in print, although recently a Gram-positive variant of *Rhizobium* was reported by Kleczkowska (1950).

In effect therefore the strains were found to be of two distinct kinds with a number of intermediate forms. They may be described as having complete and reduced life cycles respectively. The strains with a complete life cycle

(Fig. 2) notably those from lupin, sweet-pea and tree-lupin, produced swarmers very freely from large barred (i.e. multicellular) bacilli from which were also produced resistant resting cells resembling endospores. The bacteroid stages, whether in nodules or in appropriate culture media, were also multicellular and freely branched. The reduced life cycle was found in varying degrees in most of the remaining strains, and was well seen in those from cultivated peas and most species of clover. The bacteroids were roughly spherical, triangular or irregular in outline, and were either unicellular or consisted of one large cell with a side branch of a few relatively tiny cells. In normal culture the small bacteria alone were seen; often they were so small as to resemble swarmers, and presumably served as such (Bisset & Hale, 1951). The entire picture of the reduced cycle was suggestive of the degeneracy often associated with parasitism. A small proportion of strains was intermediate in morphology, and produced rare rather small endospores but seldom swarmers. The bacteroids were also intermediate in morphology in most cases.

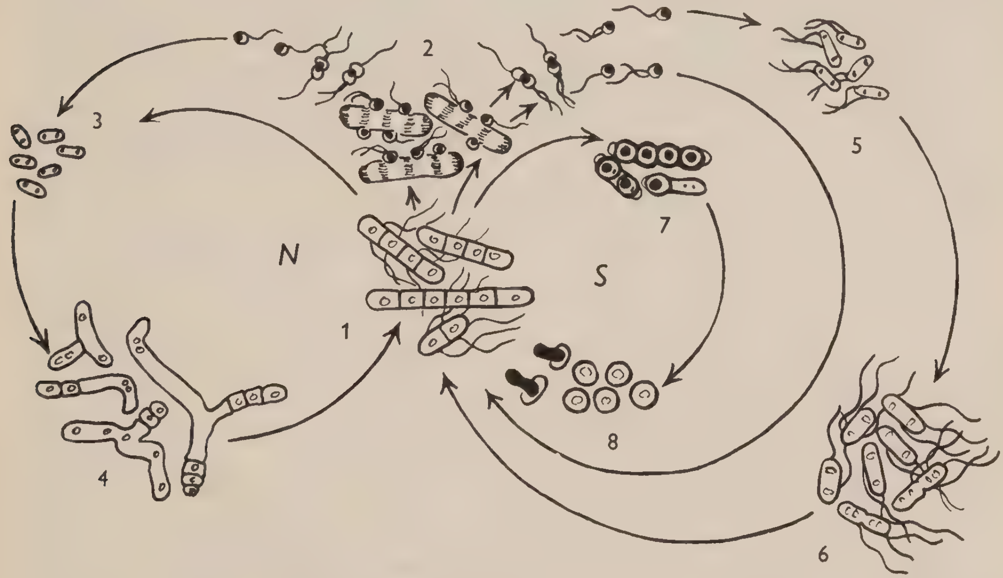


Fig. 2. Complete type of life cycle in *Rhizobium*. *N*: in the nodule, small bacteria develop into branched, multicellular bacteroids; *S*: in the soil, reproduction may be by the formation of endospores or of swarmers from large Bacillus-like rods. The swarmers are transformed into small rods which may attack the root-hairs of susceptible plants or grow up into a new generation of large bacilli, either directly or via a Bacterium-like generation. (1) Bacillus-like mother cells; (2) formation of swarmers; (3) plant-infective cells; (4) bacteroids; (5, 6) Bacterium-like motile cells; (7, 8) spore formation.

The morphology of the various strains of *Rhizobium* which were examined, and the differences between them, suggest that the genus fundamentally resembles the sporing bacilli in almost all respects, but has suffered a varying degree of loss of these characters, so that Gram-positivity is greatly reduced in all strains, some sporulate very seldom or not at all, and some, by chance the agriculturally important and best-known strains, have become so altered, presumably by prolonged parasitism, that the original morphology has been almost completely lost. It is also interesting to consider that the very typical *Rhizobium* character of reproduction by swarmers is closely paralleled by the mode of production of tiny, bacillary gonidia in many species of sporing bacillus (Bisset, 1950), although much less is known about these.

Apart from this phenomenon, *Rhizobium* appears to bear the closest resemblance to the *B. polymyxa* group, in habitat, biochemistry, tendency to loss of Gram-positivity, and even in such details as the morphology of the spore (Bisset, 1952*b*, p. 48), and the appearance of cultures.

This study may perhaps be claimed to be one of the first in which the principle of establishing the phylogeny of a genus by reference to the life cycle, so commonly employed in other biological fields, has been applied to bacteria.

REFERENCES

ALLEN, E. K. & ALLEN, O. N. (1951). Biochemical and symbiotic properties of rhizobia. *Bact. Rev.* **14**, 273.

BISSET, K. A. (1950). *The Cytology and Life-History of Bacteria.* Edinburgh: Livingstone.

BISSET, K. A. (1952*a*). Observations on the morphology and natural relationships of *Rhizobium*. *J. gen. Microbiol.* **6**, i.

BISSET, K. A. (1952*b*). *Bacteria.* Edinburgh: Livingstone.

BISSET, K. A. & HALE, C. M. F. (1951). The production of swarmers in *Rhizobium*. *J. gen. Microbiol.* **5**, 592.

KLECZKOWSKA, J. (1950). A study of phage-resistant mutants of *Rhizobium trifolii*. *J. gen. Microbiol.* **4**, 298.

LEWIS, I. M. (1938). Cell inclusions and life-cycle of rhizobia. *J. Bact.* **35**, 573.

NUTMAN, P. S. (1952). Root-nodules of leguminous plants. *J. gen. Microbiol.* **6**, i.

EXPLANATION OF PLATE

(All photomicrographs at × 3000.)

Fig. 1. *Rhizobium* sp. from lupin. Large Bacillus-like rods, showing numerous cross-walls. Tannic acid-violet.

Figs. 2 and 3. As fig. 1; showing nuclear structures. Acid Giemsa.

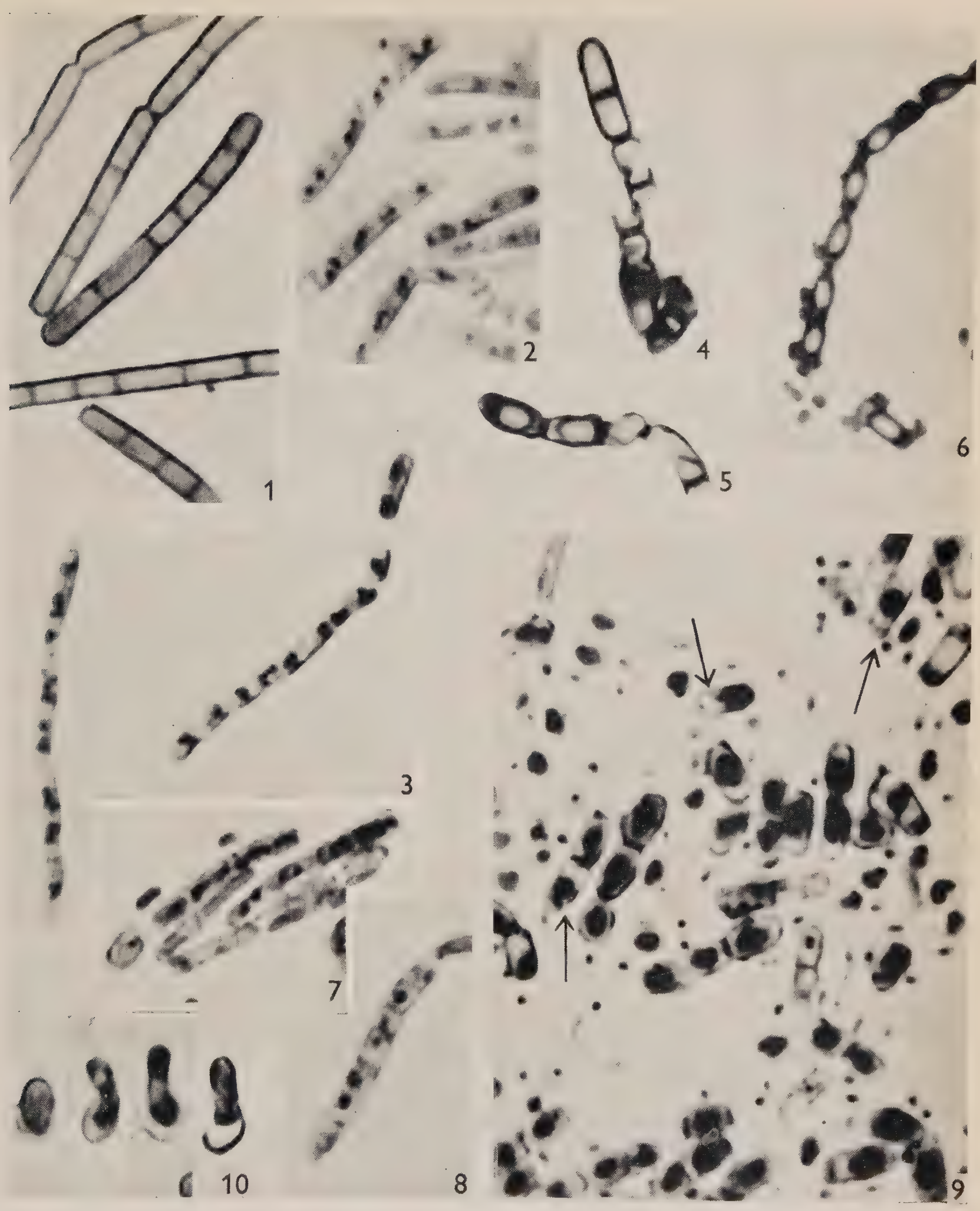
Figs. 4 and 5. As fig. 1: liberation of endospores by rupture of the cell wall. Cross-walls well seen. Tannic acid-violet.

Fig. 6. As fig. 1: chains of endospores. Gram.

Figs. 7–9. As fig. 1: stages in spore formation. In fig. 7 the rod-shaped fusion nuclei are seen; in fig. 9 the arrows indicate stages in the reduction process and elimination of the rejected nucleoid. Acid Giemsa.

Fig. 10. Germination of spores.

(*Received* 10 *March* 1952)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K. A. BISSET—LIFE CYCLES IN *RHIZOBIUM*. PLATE 1

Stanton, W. R. (1952). *J. gen. Microbiol.* 7, 243–244.

A Simple Chamber for Use in Aseptic Microdissection

By W. R. STANTON

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SUMMARY: A Perspex housing for a binocular microscope is described and the method is given for the production and maintenance of sterility inside it.

In embryo culture, tissue culture and microbiological work, there is often a need for simple chambers for aseptic working and a number of designs have been used. Many suffer from over elaboration in an attempt to maintain complete sterility in their interiors. Such chambers may become white elephants in the laboratory because of the time required to prepare them for use and their awkwardness. Cleaning and lighting also present problems. This note describes a simple chamber which overcomes some of these problems.

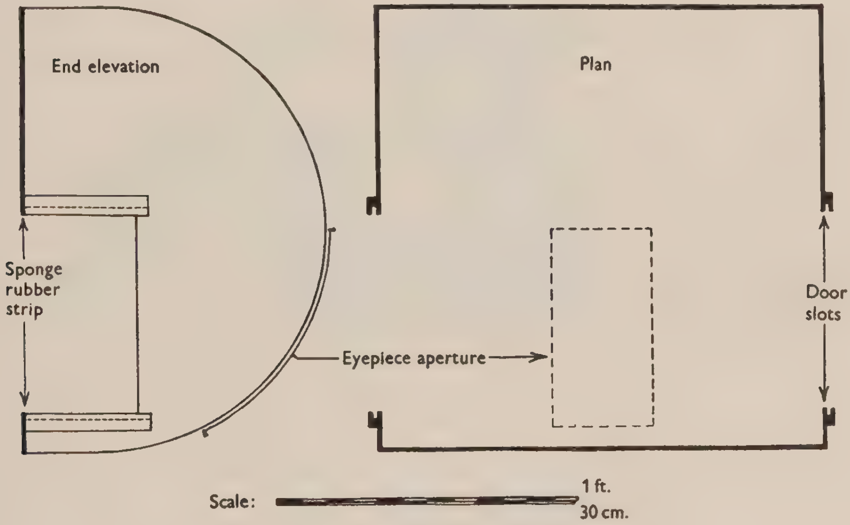


Fig. 1. Plan of chamber.

The chamber, illustrated in Pl. 1 and Fig. 1, is made of $\frac{3}{16}$ in. Perspex with two holes for the arms which can be closed by sheets of the same material. A strip of sponge rubber around the base of the chamber provides a seal between it and the sheet of plate glass on which it is mounted. The eyepiece aperture is designed so that the microscope can be used with or without a foot in an inclined or vertical position. It is closed with sheet polythene, though other plastics would be suitable, and to this sheet are fixed two tubes of the same material which are crimped on to the eyepieces by rubber bands.

Sterility is initially produced by spraying the interior with thymol (0·1 %) in ethanol. The sterilizing agent used will vary with the material being dissected, but solvents of Perspex and substances which leave a hygroscopic or gummy residue must be avoided. The principles of aerial sterilization and the properties of different sterilizing agents were discussed by Bourdillon and others (1948). Humidity, an important adjunct in keeping the aerial population low, is maintained by a cylinder of filter-paper in a small dish of water. With the chamber closed under these conditions, agared slides on the microscope stage will stay moist for long periods.

The flaming of instruments *outside* the chamber avoids convection currents and the still air in the chamber is relatively little disturbed by movement of the hands in the course of manipulation. Petri dishes, which in the laboratory would be contaminated by opening sufficiently to plant tissue in the ordinary way, can remain uncovered and free from contamination in the chamber even after it has been in use.

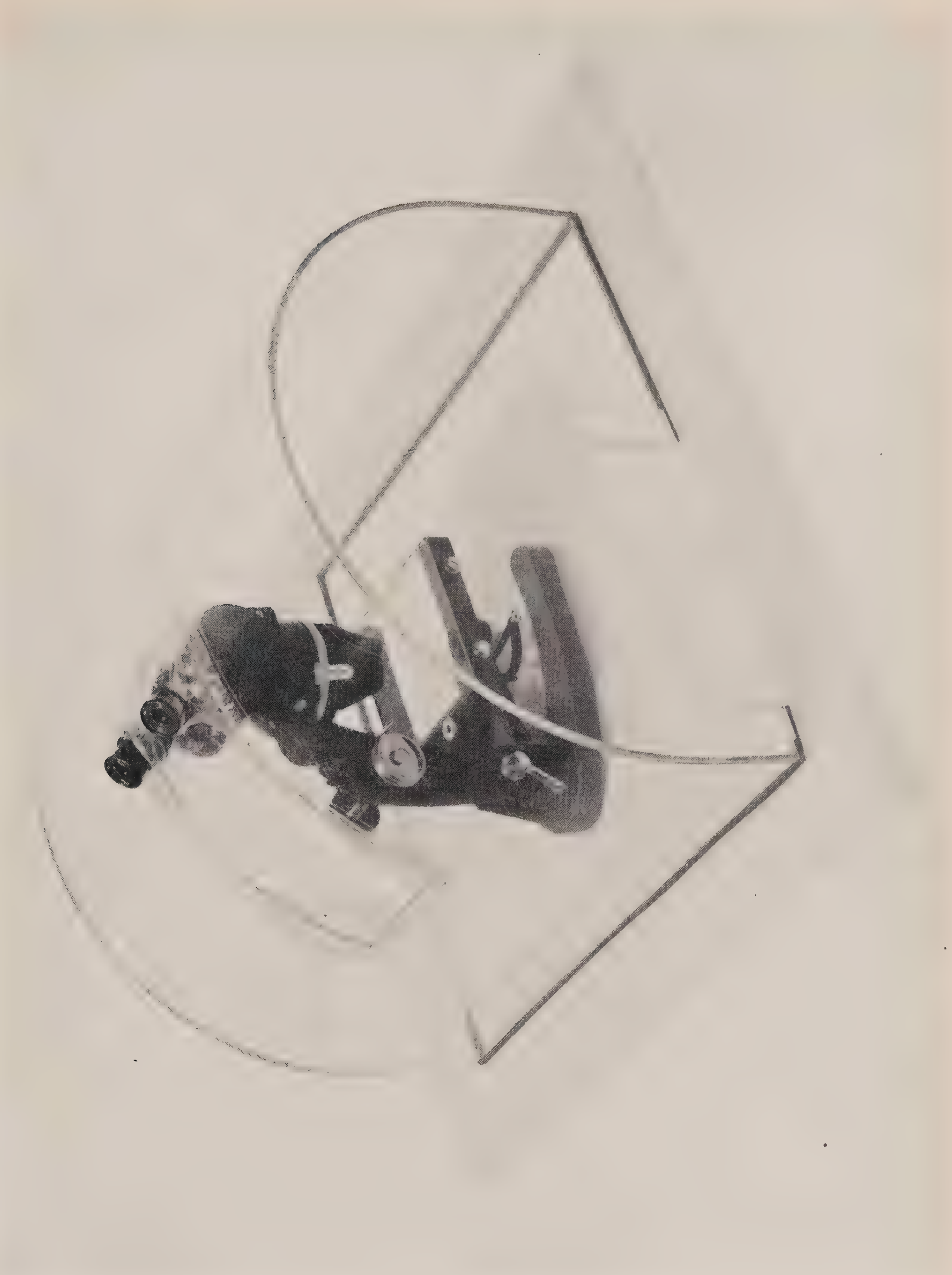
REFERENCE

Bourdillon, R. B. (1948). Studies in air hygiene. *Spec. Rep. Ser. med. Res. Coun., Lond.* no. 262.

EXPLANATION OF PLATE

Fig. 1. Chamber fitted over microscope.

(*Received* 19 *March* 1952)



W. R. STANTON—A SIMPLE MICRODISSECTION CHAMBER. PLATE 1

NORTHAM, B. E. & NORRIS, F. W. (1952). *J. gen. Microbiol.* 7, 245–256.

A Microbiological Assay of Inositol: its Development and Statistical Analysis

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SUMMARY: The assay of inositol has been developed using *Kloeckera brevis* B 768 and a strain of *Schizosaccharomyces pombe*. In the assay of Difco Bacto yeast extract it was necessary to supplement the basal medium with an inositol-free preparation from yeast extract. A new assay design has been proposed and the results treated statistically.

A microbiological assay of inositol was described by Woolley (1941) using a strain of *Saccharomyces cerevisiae*. This was based on the fact that the rate of growth of the organism is stimulated by inositol; but it has the disadvantage that the organism can grow in the absence of an exogenous supply of inositol. A similar method is given by Williams, Stout, Mitchell & McMahon (1941).

Burkholder, McVeigh & Moyer (1944) described an assay method using *Kloeckera brevis*, and Emery, McLeod & Robinson (1946) have given standard curves for *K. brevis* and *Schizosaccharomyces pombe*. These organisms appeared to be most suitable for further study since they were unable to grow in the absence of inositol even after prolonged incubation.

Although the inositol content of Difco Bacto yeast extract (DYE) was in itself of small importance it was chosen as suitable test material in the development of the assay since it is rich in substances that stimulate the growth of yeast. Hence a reliable method for the assay of yeast extract might also be expected to give reliable results when applied to other complex biological materials.

EXPERIMENTAL

Tube tests. The usual procedure for microbiological assay was followed, but in place of bacteriological tubes, flat-bottomed tubes (Samco), $4 \times \frac{5}{8}$ in., were used. Tubes were selected so that their internal diameter was within the limits 1·66 and 1·72 cm.

Test and standard solutions were added to the tubes from a 5 ml. microburette, and the volume made up to 3 ml. with water; 3 ml. of basal medium at double strength was added by means of an automatic pipette, similar in construction to that described by Ridyard (1949).

The tubes containing 6 ml. of test medium and covered with loosely fitting glass caps were sterilized in the autoclave by heating until 10 lb. pressure was reached, at which point the gas was extinguished and the tubes allowed to cool. After inoculation, they were incubated in a water-bath at 25° ± 0·2°.

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* Formerly Industrial Fermentation.

Estimation of amount of yeast growth

(*a*) When studying growth rate: the turbidity of the yeast suspension was measured by fitting the tubes into a wooden container placed in the optical beam of the Spekker absorptiometer (Northam & Norris, 1951).

(*b*) When measuring growth for an assay: the yeast was brought into an even suspension by swirling the medium in the tubes and then pouring into a 1 cm. glass cell. The turbidity was measured in the Spekker absorptiometer fitted with neutral grey-green filters no. H 508.

Dilution unit-turbidity calibration. The drum readings (turbidity) obtained using the Spekker were plotted against the cell concentration of yeast suspensions (dilution units) prepared by the dilution of portions of cultures grown under the usual experimental conditions.

Maintenance of cultures. K. brevis and *S. pombe* were subcultured monthly, or more frequently as required. The organisms were carried on malt agar slopes supplemented with a yeast extract, such as Difco Bacto yeast extract, at the rate of 2 g./l.

Inoculum. A loopful of a 24 hr. culture was suspended in 6 ml. of basal medium to give a turbidity in the tubes reading between 0·15 and 0·25 in the Spekker (i.e. approximately 0·2–0·4 mg. dry weight of yeast/6 ml.). For inoculation one drop (20 μl.) of the suspension was added to each tube from a sterile Pasteur pipette.

Development of the basal medium

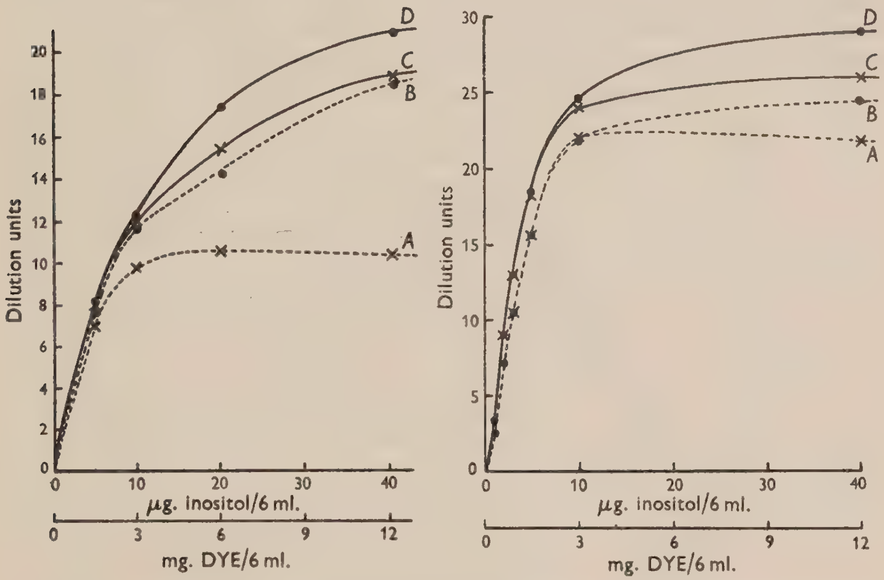
Unlike the other B vitamins, inositol is required in relatively large amounts by certain micro-organisms so that in the assay of a test preparation considerable amounts of other substances will be added to the assay medium Consequently it is important that the basal medium should be as complete as possible with respect to these substances (Woolley, 1947). It was found that the medium of Emery *et al.* (1946) could be improved by increasing the concentration of vitamins and adding casein hydrolysate (CH). Since test substances often have strong buffering effects, it was considered that the basal medium itself should be well buffered. Lactate buffer was found to be satisfactory for this purpose.

The modified basal medium, BMI + CH, corresponds to 'Basal medium III' described by Northam & Norris (1951) with the exceptions that the lactate buffer was adjusted to pH 4·8 and the concentration of the casein hydrolysate was diminished by one half.

Evidence that the basal medium BMI + CH was deficient in certain stimulatory substances required by *K. brevis* is given in Fig. 1*a*. Here the weight of yeast formed after 72 hr. incubation at a level of 12 mg. DYE/6 ml. of medium was approximately 80 % greater than that formed in the presence of an optimal concentration of inositol (curves *A* and *B*). The rate of growth of *K. brevis* was also markedly stimulated by DYE.

In the case of *S. pombe*, DYE had little effect on the rate of growth and increased the final extent of growth by only 10 % (Fig. 1*b*, curves *A* and *B*).

As might be expected from these considerations, the result of the assay of inositol in DYE obtained using *S. pombe* (3·59 mg./g.) was lower than that given by *K. brevis* (3·72 mg./g.) (Table 4). In these circumstances the assay results could not be regarded with confidence, and therefore it was considered desirable to supplement the basal medium with an inositol-free preparation of yeast extract which would supply the unknown stimulatory substances.



a *b*

Fig. 1. Effect of the supplement on the response of (*a*) *K. brevis* and (*b*) *S. pombe* to inositol and DYE. - - - - -, BMI+CH; ———, BMI Supp.; ×, inositol; ●, DYE.

Preparation of the supplement. As only a limited amount of DYE was available, a yeast extract produced by Norman Evans and Rais Ltd. (NEYE) was used.

Of the methods of fractionation attempted (Northam, 1951) the following was found to be satisfactory:

9·6 g. NEYE were stirred for 10 min. with a mixture of 760 ml. absolute ethanol and 40 ml. 2N-NaOH. After centrifuging, the extract was neutralized with 5N-HCl and filtered through Whatman no. 1 filter-paper. The ethanol was removed by evaporating almost to dryness *in vacuo* at 30°. The residue was taken up in distilled water, adjusted to pH 4·8, filtered on Whatman no. 44 filter-paper, and made up to 100 ml. This supplement was added to the basal medium at the rate of 83 ml./l.

Effect of the supplement on the growth of the organism

At the lower levels of inositol (0–5 μg./6 ml.) the growth of *K. brevis* reached a limit within 24 hr. in the presence of the supplement compared with 48 hr. in the unsupplemented medium. Moreover, the rate of growth in the presence of the test preparation (DYE) was the same as that at corresponding levels of the standard preparation (Fig. 2). This suggests that in the presence of the supplement, DYE is acting merely as a dilute solution of inositol, and that accurate assay results could be obtained at any time during the growth of the organism. The effect of the supplement on the final extent of growth of *K. brevis* and *S. pombe* is shown in Fig. 1*a*, *b* respectively. It was shown that the addition of casein hydrolysate to the supplemented basal medium had no effect on the growth of either organism at various levels of inositol. It was therefore omitted from the basal medium.

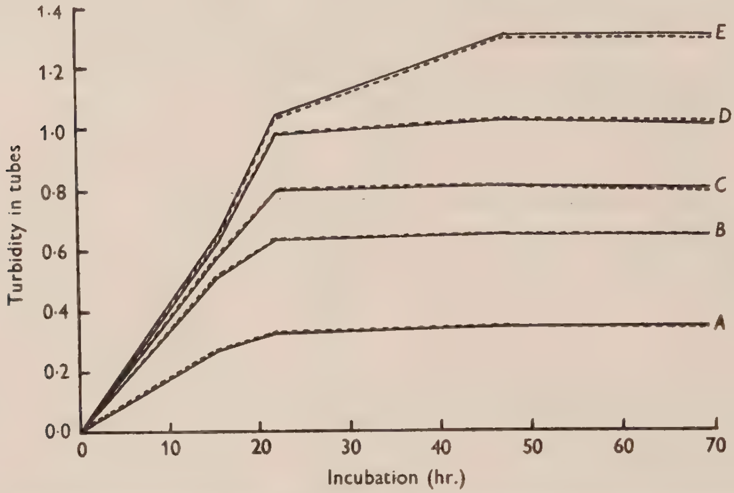


Fig. 2. Comparison of the response of *K. brevis* to inositol and corresponding levels of DYE in the supplemented basal medium. ———, $A=1{\cdot}0$; $B=2{\cdot}0$; $C=3{\cdot}0$; $D=5{\cdot}0$; $E=10{\cdot}0$ μg. inositol/6 ml. - - - - -, $A=290$; $B=580$; $C=870$; $D=1450$; $E=2900$ μg. DYE/6 ml.

Design and analysis of the assay

In order to compare assay values for the amount of inositol in Difco Bacto yeast extract (DYE) under the various experimental conditions described it was desirable to ascribe standard errors to these results. The need for the statistical analysis of microbiological assays has recently been emphasized by Finney (1951*a*). Such calculations can only be carried out conveniently when the response, or a metameter of the response, is a linear function of the dose. When the response was measured as the turbidity of the yeast suspension, the dose-response relationship was not linear. Log transformations did not help and other methods were sought.

(1) While there was reason to believe that a linear relationship existed between the weight of yeast formed and the dose, it was impracticable to weigh the yeast directly and a dilution method had to be used. Weight-turbidity transformation curves were constructed by plotting dilution of yeast suspension (grown on a suitable medium) against the turbidities of these dilutions. Certain disadvantages however were inherent in the method:

(*a*) The weight-turbidity relation of yeast grown at a concentration of, say, 40 μg. inositol/6 ml. was not the same as that grown at 2 μg./6 ml.

(*b*) Since the shape of the standard curve varied significantly from one experiment to another, it was not possible to apply a fixed transformation curve to a series of assays.

While this method gave satisfactory results on some occasions, on others there was a significant deviation from linearity which may have been due to fundamental faults in the assay, or to a systematic error in the construction of the transformation curve.

(2) A method of transformation first suggested by Ipsen (1941) and later by Finney (1947*a*, *b*) avoids the errors inherent in the construction of a weight-turbidity transformation curve. In this method, an accurately determined standard curve is used as the transformation curve and the response in subsequent assays expressed in 'dose-equivalents' by reading off the doses corresponding to those responses from the transformation curve. Thus if a standard curve has the same shape as the transformation curve, then when the response is expressed in dose-equivalents, the regression of dose-equivalents upon dose will be linear and will pass through the origin.

Furthermore, if the test curve has the same shape as the standard curve (and this must be so if the assay is valid; Wood, 1945; Finney, 1945), then it will also become a straight line passing through the origin and the ratio of the slopes of the test and standard lines will give an estimate of the relative potency of the preparation. The deviations from linearity will provide a measure of the validity of the assay. However, the method does not overcome the difficulty that when the shape of the standard curve changes from one experiment to another the transformation curve is no longer applicable.

(3) For this reason, it was considered preferable to prepare a transformation curve with each assay. The usual standard curve can in fact, with suitable modification to the statistical analysis, be used as the transformation curve. If this curve is to be drawn by eye, gross errors must be avoided by arranging that the test and standard responses are near the same level. However, the disadvantages of this procedure may be avoided by having two points (referred to subsequently as a 'Bracket') close together on the standard curve on either side of the test responses. The response may then be expressed in dose-equivalents by simple calculation from the slope of the line joining these two points (the slope is given by y/x in Figs. 3 and 5). The distance between these points is governed by two considerations, viz. (*a*) they should be sufficiently far apart to permit an accurate determination of the slope between them; and (*b*) they should be sufficiently close together for a straight line joining the points to deviate but slightly from the true curve.

The transformed data may be analysed according to the method of multiple regression given by Burn, Finney & Goodwin (1950) with certain modifications to the statistical treatment (described below).

It must be emphasized, however, that this design and analysis can only be expected to give a close approximation to the correct result when the replication is good and the dose-response curve of a regular shape (Finney, 1951, private communication). The method described above for the design and analysis of assays will be referred to as the 'bracket transformation' method.

Worked example of the 'bracket transformation' method

The assay of DYE using *S. pombe* in the supplemented basal medium, BMI Supp., is here described as an example of the application of the bracket transformation method given above. The turbidity was measured in a ·1 cm. Spekker cell after 72 hr. incubation. The relationship between dose and response is shown in Table 1 and Fig. 3. The turbidities of the replicates at each test level were expressed in dose-equivalents by simple calculation from the slopes of the appropriate brackets, and the replicates at each standard level were treated in the same way. Thus a turbidity 'y' may be expressed in dose-equivalents 'Y' from the equation

Table 1. *Response of* S. pombe*—turbidity measured in Spekker cell—to various doses of inositol and DYE after 72 hr. incubation in the supplemented basal medium BMI Supp.*

One unit of standard preparation (x_s) = 0·25 μg. inositol.
One unit of test preparation (x_t) = 75 μg. DYE.

	Spekker readings						
Units inositol	Standard curve						Mean
3·6	0·172	0·160	0·160	0·169	0·179		0·168
4·4	0·309	0·310	0·307	0·310	0·309		0·309
5·6	0·477	0·485	0·488	0·475	0·479		0·481
6·4	0·568	0·580	0·577	0·589	0·575		0·578
7·6	0·710	0·702	0·718	0·713	0·708		0·710
8·4	0·790	0·787	0·798	0·798	0·782		0·791
Units DYE	Test curve						
4·0	0·261	0·270	0·257	0·290	0·270	0·269	0·269
6·0	0·560	0·563	0·560	0·562	0·565	0·566	0·563
8·0	0·779	0·787	0·775	0·786	0·782	0·786	0·782

$$Y = x_1 + (y - y_1)\left(\frac{x_2 - x_1}{y_2 - y_1}\right),$$

where x_1, y_1 are the co-ordinates of the lower point and x_2, y_2 are the co-ordinates of the upper point of the standard bracket.

For example, the dose-equivalent corresponding to a turbidity of 0·261 at the lowest test level (Table 1) was given by the equation

$$Y = 3{\cdot}6 + (0{\cdot}261 - 0{\cdot}168)\left(\frac{(4{\cdot}4 - 3{\cdot}6)}{(0{\cdot}309 - 0{\cdot}168)}\right).$$

The transformed data are given in Table 2 and Fig. 4.

These data were analysed by the method of multiple regression (Burn *et al.* 1950, pp. 97–101) and the symbols used here are the same as those adopted by these authors.

Table 2. *Response of* S. pombe *expressed in dose-equivalents*

One unit of standard preparation (x_s) = 0·25 μg. inositol.
One unit of test preparation (x_t) = 75 μg. DYE.

Standard	x_s	x_s^2	y_s – dose-equivalents					Σy_s	$\Sigma x_s y$
	3·6	12·96	3·62	3·55	3·56	3·61	3·66	18·0	64·8
	4·4	19·36	4·40	4·41	4·38	4·41	4·40	22·0	96·8
	5·6	31·36	5·57	5·63	5·66	5·55	5·59	28·0	156·8
	6·4	40·96	6·32	6·42	6·39	6·49	6·38	32·0	204·8
	7·6	57·76	7·60	7·52	7·68	7·62	7·58	38·0	288·8
	8·4	70·56	8·39	8·36	8·47	8·47	8·31	42·0	352·8
Totals	36·0	232·96	—	—	—	—	—	180·0	1164·8

Test	x_t	x_t^2	y_t – dose-equivalents						Σy_t	$\Sigma x_t y$
	4·0	16·0	4·13	4·18	4·10	4·29	4·18	4·17	25·05	100·20
	6·0	36·0	6·26	6·28	6·25	6·27	6·30	6·31	37·67	226·02
	8·0	64·0	8·28	8·36	8·24	8·35	8·31	8·35	49·89	399·12
Totals	18·0	116·0	—	—	—	—	—	—	112·61	725·34

$\bar{x}_s = 180$ $\Sigma x_s^2 = 1164{\cdot}8$ $\Sigma x_s y = 1164{\cdot}80$ $\Sigma y = 292{\cdot}61$

$\bar{x}_t = 108$ $\Sigma x_t^2 = 696{\cdot}0$ $\Sigma x_t y = 725{\cdot}34$ $\Sigma N = 48$

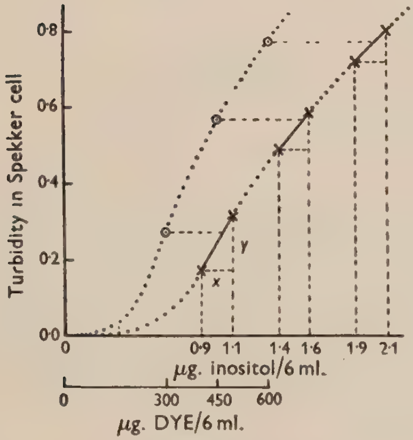


Fig. 3

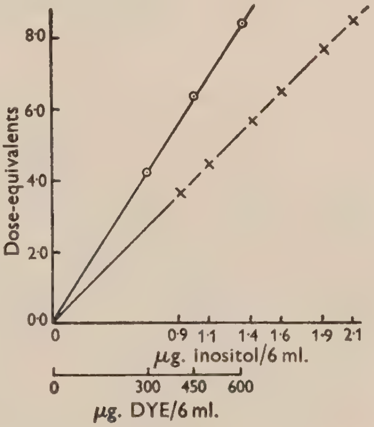


Fig. 4

Fig. 3. Response of *S. pombe* (turbidity measured in the Spekker cell) to various doses of inositol and DYE after 72 hr. incubation in BMI Supp. ×, inositol; ⊙, DYE.

Fig. 4. Response of *S. pombe* expressed in dose-equivalents. ×, inositol; ⊙, DYE.

Multiple regression. The test and standard lines may be represented by the bivariate equation

$$Y = a + b_s x_s + b_t x_t,$$

where x_s (the dose of the standard preparation) is zero for all doses of the test preparation and x_t (the dose of the test preparation) is zero for all doses of the standard. The regression coefficients may be evaluated from the equations

$$\left.\begin{aligned} b_s S x_s x_s + b_t S x_s x_t &= S x_s y, \\ b_s S x_s x_t + b_t S x_t x_t &= S x_t y, \end{aligned}\right\} \qquad \text{(i)}$$

where
$$
\begin{aligned}
Sx_sx_s &= \Sigma x_s^2 - (\Sigma x_s)^2/N\\
&= 1164{\cdot}8 - (180)^2/48 = 489{\cdot}8,\\
Sx_sx_t &= \Sigma x_sx_t - (\Sigma x_s)\,(\Sigma x_t)/N\\
&= 0 - (180)\,(108)/48 = -405,\\
Sx_tx_t &= \Sigma x_t^2 - (\Sigma x_t)^2/N\\
&= 696{\cdot}0 - (108)^2/48 = 453,\\
Sx_sy &= \Sigma x_sy - (\Sigma x_s)\,(\Sigma y)/N\\
&= 1164{\cdot}8 - (180)\,(292{\cdot}61)/48 = 67{\cdot}5125,
\end{aligned}
$$
and
$$
\begin{aligned}
Sx_ty &= \Sigma x_ty - (\Sigma x_y)\,(\Sigma y)/N\\
&= 725{\cdot}34 - (108)\,(292{\cdot}61)/48 = 66{\cdot}9675.
\end{aligned}
$$

Substituting these values in equations (i) gives
$$b_s = 0{\cdot}9974177, \qquad b_t = 1{\cdot}0395621.$$
The equation for 'a' is given by
$$
\begin{aligned}
a &= \bar{y} - b_sx_s - b_tx_t\\
&= 292{\cdot}61/48 - (0{\cdot}9974177 \times 3{\cdot}75) - (1{\cdot}0395621 \times 2{\cdot}25)\\
&= 0{\cdot}0167106.
\end{aligned}
$$
The multiple regression equation therefore becomes
$$Y = 0{\cdot}0167106 + 0{\cdot}9974177\ x_s + 1{\cdot}0395621\ x_t.$$

Analysis of variance. The regression sum of squares is given by
$$
\begin{aligned}
b_sSx_sy + b_tSx_ty &= 0{\cdot}9974177 \times 67{\cdot}5125 + 1{\cdot}0395621 \times 66{\cdot}9675\\
&= 136{\cdot}955037.
\end{aligned}
$$

The total, between doses, and error components are obtained by the usual statistical methods. However, since the dose means of the standard preparation have been forced to be co-linear it is necessary to remove a number of degrees of freedom two less than the number of doses for the standard from the total, between doses, and deviations components of the analysis of variance (Finney, 1951*b*).

The analysis of variance is given in Table 3.

Table 3. *Analysis of variance*

Nature of variation ...	D.F.	Sum of squares	Mean square
Adjustment for mean		1,783·76275	
Regression	2	136·95504	—
Deviations from regression	2	0·00613	0·00306
Between doses	4	136·96117	—
Error	39	0·10038	0·00257
Total	43	137·06155	—

Test of validity. The ratio of the deviations mean square to the error mean square
$$F = \frac{0{\cdot}003064}{0{\cdot}002574} = 1{\cdot}19.$$

With $n_1 = 2$, $n_2 = 39$, this is clearly well below the significance level of 3·24, showing that the assay is statistically valid.

Relative potency. Unit dose of the test preparation is equal to R units of the standard, where

$$R = \frac{b_t}{b_s} = 1\cdot0422536.$$

From the definition of the units of the two preparations at the head of Tables 1 and 2, 75 μg. DYE contains 0·25 × 1·0422536 μg. inositol. Hence, 1 g. DYE contains 3·47418 mg. inositol.

Fiducial limits. The approximate variance of the potency ratio R is given by the equation

$$V(R) = \frac{s^2}{b_s^2} \times \frac{Sx_sx_s \times 2RSx_sx_t + R^2Sx_tx_t}{Sx_sx_sSx_tx_t - (Sx_sx_t)^2}.$$

This expression is applicable only if the values of g is small (say, less than 0·1) where

$$g = \frac{s^2t^2Sx_tx_t}{b_s{}^2[Sx_sx_sSx_tx_t - (Sx_sx_s)^2]}.$$

For good microbiological assays, g will always be negligible (Wood & Finney, 1946), and for this particular assay, $g = 0\cdot0001$. Thus:

$$V(R) = \frac{0\cdot002574}{(0\cdot9974177)^2} \times \frac{489\cdot8 - 810 \times 1\cdot0422536 + (1\cdot0422536)^2 \times 453}{489\cdot8 \times 453 - (405)^2}$$

$$= 0\cdot0000061566$$

$$s_R = \sqrt{[V(R)]} = 0\cdot00248$$

and

$$R = 1\cdot04225 \pm 0\cdot00248.$$

At 39 degrees of freedom and a probability of 0·05, $t = 2\cdot023$ so that the fiducial limits, 2·023 × 0·00248 on either side of R became 1·03723, 1·04727. The estimated potency, 3·474 mg. inositol/g. yeast extract, was therefore assigned fiducial limits of 3·457, 3·491 mg. inositol/g. Difco Bacto yeast extract.

A similar design and analysis was employed for assays using *K. brevis* (Figs. 5 and 6) with the modification that the dose levels of the standard preparation were 0·75, 1·05; 1·65, 1·95; 2·55, 2·85 μg. inositol/6 ml. (inositol solution made up at a concentration of 1 μg./ml.) and the test levels were 270, 540 and 810 μg./tube (DYE solution at 300 μg./ml.).

RESULTS

The results for the assay of inositol in DYE under the various conditions discussed are given in Table 4. The assay using *K. brevis* on the unsupplemented medium was computed using a dilution unit-turbidity transformation curve. Since the results were obviously significantly higher than that on the supplemented medium the assay was not repeated using the bracket transformation method.

On the supplemented medium, the assay using *K. brevis* was computed using the bracket transformation method with the six standard doses (i.e. three standard brackets) replicated six times and the three test doses replicated four times. This was modified to a more efficient design (Finney, 1951*b*) for the *S. pombe* assays with the three standard brackets replicated five times and the three test doses replicated six times.

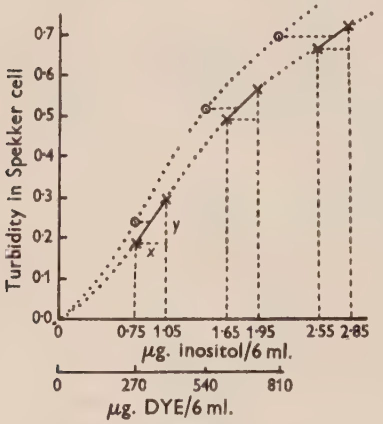


Fig. 5

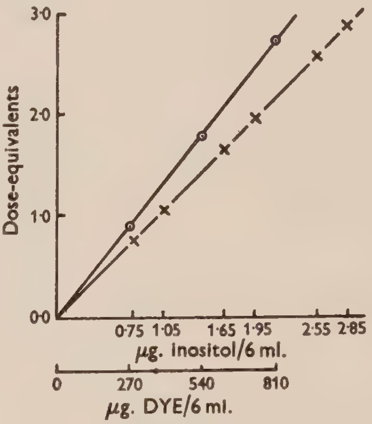


Fig. 6

Fig. 5. Response of *K. brevis* (turbidity measured in Spekker cell) to various doses of inositol and DYE after 72 hr. incubation in BMI Supp. ×, inositol; ⊙, DYE.

Fig. 6. Response of *K. brevis* expressed in dose-equivalents. ×, inositol; ⊙, DYE.

Table 4. *Comparison of assay results*

Organism	Basal medium	Period of incubation (hr.)	Potency ± S.E. Fiducial limits ($P = 0·95$)	Test of validity *F*	*P*
K. brevis	BMI Supp.	24*	3·441 ± 0·025 3·39 − 3·49	0·54	> 0·20‡
		72*	3·461 ± 0·023 3·41 − 3·51	1·32	> 0·20‡
		74†	3·441 ± 0·021 3·40 − 3·48	0·68	> 0·20‡
	BMI + CH	72†	3·723 ± 0·019 3·68 − 3·76	8·45	< 0·001
S. pombe	BMI Supp.	48*	3·462 ± 0·018 3·42 − 3·50	0·45	> 0·20‡
		70*	3·458 ± 0·016 3·42 − 3·49	0·77	> 0·20‡
		72†	3·474 ± 0·008 3·46 − 3·49	1·19	> 0·20‡
	BMI + CH	73†	3·586 ± 0·012 3·56 − 3·61	6·61	0·01–0·001

The potency is expressed in mg. inositol/g. DYE.
* Turbidity measured in the tubes. † Turbidity measured in Spekker cell.
‡ Assay statistically valid.

DISCUSSION

It is evident from Table 4 that addition of the supplement to the basal medium reduced the apparent inositol content of Difco Bacto yeast extract obtained from assays using either *K. brevis* or *S. pombe*. (While estimates of potency and fiducial limits of statistically invalid assays may not be strictly accurate it is likely that these estimates are sufficiently accurate for the purposes of comparison.) From previous considerations the lower value would be expected to give a truer estimate of the potency. The observation that assay values obtained using *K. brevis* and *S. pombe* in the supplemented medium do not differ significantly provides strong support for the reliability of assays carried out under these conditions.

The choice of organisms for subsequent assays is governed by the following considerations:

(*a*) Assays using *K. brevis* and *S. pombe* may be completed after 24 and 48 hr. respectively without loss of accuracy.

(*b*) The 95 % fiducial limits of assays using *S. pombe* were approximately ±0·5 % as compared with ±1 % for *K. brevis*.

(*c*) *S. pombe* requires less inositol than *K. brevis* to produce a given response, and although this does not affect the precision of the assay (Wood, 1946) it may be advantageous in that less test material (and therefore less interfering substances) would be added to the assay medium.

The method is being applied successfully to cereals and cereal products in these laboratories.

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REFERENCES

Barton-Wright, E. C. (1946). *Practical Methods for the Microbiological Assay of the Vitamin B Complex and Essential Amino-Acids.* London: Ashe Laboratories.

Burkholder, P. R., McVeigh, L. & Moyer, D. (1944). Studies of some growth factors of yeasts. *J. Bact.* **48**, 385.

Burn, J. H., Finney, D. J. & Goodwin, L. G. (1950). *Biological Standardization.* London: Oxford University Press.

Emery, W. B., McLeod, N. & Robinson, F. A. (1946). Comparative microbiological assays of members of the vitamin B complex in yeast and liver extracts. *Biochem. J.* **40**, 426.

Finney, D. J. (1945). The microbiological assay of vitamins: the estimate and its precision. *Quart. J. Pharm.* **18**, 77.

Finney, D. J. (1947*a*). The principles of biological assay. *J. R. statist. Soc.* (Suppl.), **9**, 46.

Finney, D. J. (1947*b*). Statistical aspects of microbiological assays. *Biochem. J.* **41**, v.

Finney, D. J. (1951*a*). The statistical analysis of slope-ratio assays. *J. gen. Microbiol.* **5**, 223.

Ipsen, J. (1941). *Contributions to the Theory of Biological Standardization.* Copenhagen: Nyt Nordisk Forlag, Arnold Busck.

Northam, B. E. (1951). *The Microbiological Assay of Inositol.* Ph.D. thesis, Birmingham University.

Northam, B. E. & Norris, F. W. (1951). Growth requirements of *Schizosaccharomyces octosporus*, a yeast exacting towards adenine. *J. gen. Microbiol.* **5**, 502.

Ridyard, H. N. (1949). High accuracy and high speed pipettes and associated apparatus. *Analyst*, **74**, 24.

Williams, R. J., Stout, A. K., Mitchell, H. K. & McMahon, J. R. (1941). Assay method for inositol. *Univ. Texas Publ.* no. 4137, 27.

Wood, E. C. (1945). Calculation of the results of microbiological assays. *Nature, Lond.* **155**, 632.

Wood, E. C. (1946). The theory of analytical procedures with special reference to microbiological assays. *Analyst*, **71**, 1.

Wood, E. C. & Finney, D. J. (1946). The design and statistical analysis of microbiological assays. *Quart. J. Pharm.* **19**, 112.

Woolley, D. W. (1941). A method for the estimation of inositol. *J. biol. Chem.* **140**, 453.

Woolley, D. W. (1947). The determination of inositol. *Biol. Symp.* **12**, 279.

(*Received* 19 *March* 1952)

Allen, L. A., Pasley, S. M. & Pierce, M. S. F. (1952). *J. gen. Microbiol.* 7, 257–267.

Conditions affecting the Growth of *Bacterium coli* on Bile Salts Media. Enumeration of this Organism in Polluted Waters

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SUMMARY: The growth of *Bacterium coli* on agar media containing bile salts is conditioned by a number of factors. The proportion of total cells able to grow on a medium not containing bile salts rapidly declines at temperatures of incubation above 43°. On a medium containing bile salts and lactose a distinctly inhibitory effect is observed at 37°; with most strains this effect is still more pronounced at 44°. Some brands of bile salts are appreciably more inhibitory than others. Inclusion of phosphate in a bile salts medium introduces a markedly inhibitory factor, the severity of which varies with the strain of organism; some strains are virtually unable to grow on such a medium.

When a culture of *Bact. coli* is suspended in water containing only small concentrations of inorganic salts an increasing proportion of the population becomes attenuated so that the cells are unable to grow on bile salts lactose agar at 44°. An occasional strain may also exhibit sensitiveness to the presence of neutral red. This attenuating effect may be largely decreased if, before the inoculum is mixed with the bile salts and agar, it is subjected to a short period of incubation with lactose broth. This treatment has been made the basis of a technique for obtaining a colony count of *Bact. coli* which is applicable to polluted waters.

Several workers (cf. Wilson, Twigg, Wright, Hendry, Cowell & Mair, 1935; Clegg & Sherwood, 1939; Sherwood & Clegg, 1942; Batty-Smith, 1942; Allen, Brooks & Williams, 1949), have found ability to ferment bile salts lactose broth at 44° a reasonably specific test for *Bacterium coli* type I, and a method of enumeration based on this test was outlined by the Ministry of Health (1939). Disadvantages of the method are the large quantity of medium required and the large error involved in the computation of the most probable number. Thus Halvorson & Ziegler (1933) calculated that, with five tubes of medium to each dilution, the count obtained will be between 70 % below and 260 % above the true value.

Enumeration of this organism provides a simple method of following the effect of different treatments of sewage on the numbers of faecal bacteria discharged into a river and of the rate at which these numbers decrease as the organisms are carried downstream. A colony count of *Bact. coli* would introduce into sanitary surveys of this character a welcome improvement in accuracy. Clegg & Sherwood (1947) introduced a roll-tube method of counting faecal coli in shellfish in which inocula were mixed with a modified MacConkey agar and the tubes were incubated at 44°. Preliminary tests showed, however, that the ability of cells of *Bact. coli* to form colonies on bile salts lactose agar was profoundly affected by a number of conditions. These conditions were investigated before the results were embodied in the method of enumeration finally adopted.

EXPERIMENTAL

After various methods of preparing films of agar media in tubes and bottles had been tried a technique similar to that described by Tai & van Heyningen (1950) was adopted. A chuck, rotating horizontally, was mounted on the shaft of a variable speed motor so that a bottle containing molten nutrient medium and an inoculum could be rotated at a speed of several hundred revolutions/min. For inocula of 1 ml. small glass bottles of the type described by Proom & Hemmons (1949) with a capacity of 30 ml. were used. For a larger size of inoculum bottles measuring about 4 cm. diameter and 15 cm. in length from base to shoulder were found suitable. Details as to the kind and quantity of medium and size of inoculum are given later but, in general, the inoculum was added to a bottle containing the liquid medium at a temperature of about 48°. The bottle was gently shaken to mix the contents and immediately placed in the chuck and rotated. The chuck and bottle were arranged to project over a sink so that, while rotating, the bottle could be cooled with a jet of cold water. Four similar machines were arranged side by side. Water cooling was found to be preferable to air cooling in that it ensured a firm adherent film after the bottle had been rotated for a comparatively short time (for the small bottles about 30 sec. sufficed). With air cooling several minutes are required on a warm day and if the bottles are removed from the chuck a little too soon the agar film develops slight wrinkles and usually collapses before or during incubation.

Comparative counts in bottles and Petri dishes and with different brands of agar

Comparative trials with Petri dishes and bottles, and with New Zealand and Japanese agars, showed that neither the kind of agar nor the type of container made any significant difference to the counts. Since, owing to the firmer gel it forms, there are advantages in using New Zealand agar for bottle counts, this material was used in the subsequent experiments. In most experiments bottle counts were averages of three or four determinations.

Effect of temperature of incubation on the count

The effect of temperature of incubation on the proportion of the total population of cells able to proliferate was tested on nutrient agar (without bile salts). Six strains of *Bact. coli* were grown in nutrient broth at 37°. After incubation for 6 hr., and again after 3 days, replicate counts were made in bottles of nutrient agar at 37, 39, 40, 41, 42, 43, 44 and 45°. Counts, expressed as percentages of the corresponding counts at 37°, are shown graphically in Fig. 1 (only three of the strains were tested at temperatures up to 41°).

It is clear that a temperature of 44° is critical for this organism (cf. Clegg &

Sherwood, 1939; Taylor, 1945), and it is important that the temperature of the water-bath should be accurately controlled. The average of the twelve counts at 44° was 74 %, and of the eleven counts at 43°, 90 % of the count at 37°.

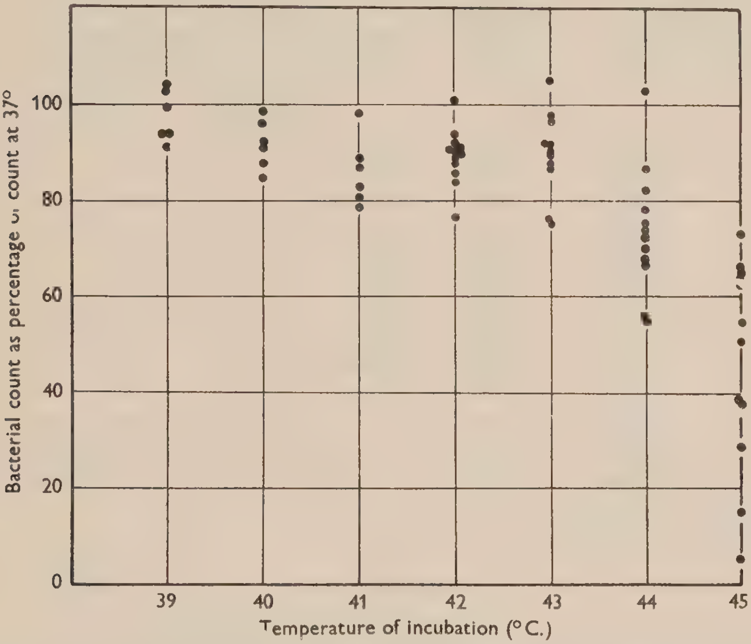

Fig. 1. Colony counts of broth cultures (when 6 hr. and when 3 days old) of six strains of *Bact. coli* at different temperatures of incubation (each count expressed as percentage of count of the same culture at 37°).

Proportion of cells able to grow on bile salts-lactose agar at 37° and at 44°

Colony counts, at both 37 and 44°, on nutrient agar and on bile salts lactose agar prepared from two different recipes (given below) were made of broth cultures of six strains of *Bact. coli* (Table 1). The inhibiting action of the bile salts media, which was appreciable at 37°, was still more marked at 44°. Medium D, containing phosphate as well as bile salts, was inferior to medium A and at the higher temperature two strains were scarcely able to grow at all. Further tests were made with this medium in which different concentrations of bile salts were tried (0·5, 1·0, 1·5 g./l.), but no difference was observed in the inhibitory effect.

Comparison of different bile salts media

It is evident from the literature that the count of *Bact. coli* will be affected by the kind and concentration of bile salts, though it is doubtful whether this is generally appreciated. Leifson (1935) found that of the bile acids he tested deoxycholic acid exerted the strongest effect on bacterial growth, and that a number of substances exerted a strongly inhibitory action on coli-aerogenes bacteria when incorporated in a sodium deoxycholate medium. These substances included sodium chloride, acetates, propionates, butyrates and citrates,

Table 1. *Comparative colony counts of* Bact. coli *on nutrient agar at 37° and on bile salts lactose agar at 37° and at 44°*

Culture		Nutrient agar at 37°	Bile salts lactose agar			
			At 37°		At 44°	
Strain	Age		Medium A	Medium D	Medium A	Medium D
		Count (millions/ml.)				
H 7	6 hr.	332	287	259	215	210
	5 days	91	51	37	30	20
H 1	6 hr.	250	165	197	156	104
	5 days	92	75	67	73	20
H 8	6 hr.	241	163	165	118	*
	5 days	44	35	19	38	<0·1
H 6	6 hr.	235	175	145	69	50
	5 days	43	36	30	32	18
H 0	6 hr.	224	184	200	157	151
	5 days	35	28	26	22	11
H 13	6 hr.	253	185	153	102	44
	5 days	6·0	4·8	0·2	2·9	0·5

Medium A = Ministry of Health recipe. Medium D = Hajna & Perry's 'EC' recipe (plus agar and neutral red).

* Counts in triplicate tubes highly inconsistent.

and their inhibitory action was enhanced by the presence of unknown substances in meat infusion and in certain brands of peptone.

Cultures of two strains of *Bact. coli* in nutrient broth were incubated at 37°, and at intervals comparative counts in bottles at 44° were made in the following four media:

A: MacConkey agar (Ministry of Health, 1939) of the following composition (g./l.): peptone, 20 g.; sodium taurocholate, 5 g.; lactose, 10 g.; neutral red, 10 ml. of 1 % solution.

B: similar to A but containing 5 g./l. of a proprietary brand of specially pure bile salts.

C: similar to A but containing 20 g./l. of ox-gall instead of sodium taurocholate.

D: the 'EC' medium prescribed by Hajna & Perry (1943) made up with agar and containing neutral red as indicator and the same brand of bile salts as B. The composition (g./l.) was as follows: Bacto tryptose, 20 g.; NaCl, 5 g.; K_2HPO_4, 4 g.; KH_2PO_4, 1·5 g.; bile salts 'X', 1·5 g.; neutral red, 5 ml. 1 % solution.

In all media 1·5 % New Zealand agar was incorporated.

Results (Table 2) showed that medium A gave higher counts than B or C. On medium D counts were much lower than on the other three media and with strain H8 the effect was so marked that virtually no growth occurred. The concentration of bile salts was less in medium D (0·15 %) than in medium B (0·5 %). This substance by itself could not, therefore, have been the main inhibitory agent.

Inhibitory effect of phosphate in bile salts media

Counts of broth cultures of six strains of *Bact. coli* were determined when 6 hr. old, and again after 6 days, on media A and D, and on medium D from which the phosphate was omitted. Results (Table 3) show that omission of phosphate removed a strongly inhibitory factor from medium D which now gave higher counts than medium A.

Table 2. *Comparative counts of* Bact. coli *in bottles at 44° on different bile salts media*

	Strain H 1				Strain H 8			
	Bile salts media							
	A	B	C	D	A	B	C	D
Age of culture	Count (millions/ml.)							
0 hr.	0·70	0·44	0·69	0·44	0·28	0·072	0·126	0*
4 hr.	151	122	119	48	61	16·2	32·2	0
24 hr.	382	204	276	208	140	15·0	22·8	0
48 hr.	306	156	164	78	250	34·2	22·0	0
72 hr.	—	—	—	—	92	7·8	10·4	0
4 days	90	44	76	3·6	—	—	—	—
5 days	—	—	—	—	52	18·4	20·4	0
6 days	18·8	12·0	14·6	2·9	41	2·4	18·6	0
7 days	20·4	16·4	12·8	10·0	—	—	—	—

A = MacConkey agar (Ministry of Health, 1939).
B = Similar to A but 5 g. proprietary brand of specially pure bile salts/l. instead of Na taurocholate.
C = Similar to A but 20 g. ox-gall/l. instead of Na taurocholate.
D = Hajna & Perry's (1943) 'EC' medium.
* less than 10/ml.

Table 3. *Effect of phosphate on counts of* Bact. coli *on bile salts lactose agar*

Culture		Bile salts lactose agar at 44°		
		Medium A	Medium D (containing phosphate)	Medium D (without phosphate)
Strain	Age	Count (millions/ml.)		
H 8	6 hr.	530	11	700
	6 days	1·52	<0·01	1·69
H 0	6 hr.	430	380	490
	6 days	5·8	0·5	7·8
H 7	6 hr.	180	99	316
	6 days	56	10	71
H 1	6 hr.	153	62	219
	6 days	60	9·3	92
H 73	6 hr.	169	80	196
	6 days	22	1·0	25
H 6	6 hr.	154	*c.* 3	172
	6 days	40	*c.* 3·5	38

Effect of suspension in water on proportion of total cells of Bact. coli *able to grow on bile salts lactose agar*

A dilute suspension of cells was obtained by adding a small quantity of a broth culture of *Bact. coli* to ¼-strength Ringer's solution (0·0015 ml. culture/l.); this was incubated at 20° and at intervals counts were made on nutrient agar at 37°, and on bile salts lactose agar (medium A) at 37° and at 44°. This procedure was repeated with broth cultures of different ages. Counts on the bile salts medium were expressed as percentages of the corresponding counts on nutrient agar, it being assumed that this medium exerted no appreciable inhibitory effect at 37°. Results for broth cultures of different ages (Table 4) indicate that in general the longer the period of immersion in Ringer's solution the smaller was the proportion of the cell population able to grow on the bile salts medium. With a young culture immersed for 3 hr. or more the inhibitory effect was much more marked at 44° than at 37°.

Table 4. *Percentage of total population of* Bact. coli *able to grow on bile salts lactose agar at 37° and at 44° after different periods of immersion in ¼-strength Ringer's solution*

	Age of broth culture (hr.)							
Period of immersion in Ringer's solution at 20° (hr.)	17 Bile salts lactose agar at		41 Bile salts lactose agar at		65 Bile salts lactose agar at		89 Bile salts lactose agar at	
	37°	44°	37°	44°	37°	44°	37°	44°
	Counts as percentage of those on nutrient agar at 37°							
0	77·5	66·3	82·4	97·3	52·4	50·5	57·3	48·3
1	78·4	63·7	72·5	62·5	32·6	48·4	49·0	40·6
2	63·8	40·6	50·0	54·8	17·4	8·8	53·5	53·5
3	46·3	16·3	51·9	23·3	13·8	7·5	46·9	46·0
6	15·8	5·7	13·0	5·4	38·0	25·4	27·1	21·1
24	—	—	11·0	7·8	—	—	8·9	7·8

In a similar experiment (Table 5) the effects of immersion in full-strength and in ¼-strength Ringer's solution were compared. By making counts on bile salts lactose agar and on a similar medium from which the bile salts had been omitted the relative effect of the bile salts and of the high temperature of incubation (44°) was assessed. The broth culture was 41 hr. old at the time of immersion. With cells immersed in the full-strength solution bile salts exerted no very marked effect, and 60 % or more of the total population was able to grow at 44°. With cells immersed in the ¼-strength solution, on the other hand, only 25–45 % of the total population of cells was able to proliferate at 44°, and of these on the average only half could tolerate bile salts. The relatively severe action of the more dilute liquid on the bacterial cells was also reflected in the rates of death in the two solutions, counts on nutrient agar at 37° showing that at the end of the 24 hr. period the percentages of the original cells surviving were 1·6 in ¼-strength and 46·7 in full-strength Ringer solution.

Experiments with a strain of *Bact. coli* in 0·003 M phosphate buffer at pH 6·0 showed that before suspension in this solution 90–100 % of cells were able to grow at 44°. After immersion for 7–12 days, however, only about one-half, and after 24 days only about one-third, of the cells could proliferate at this temperature. The temperature of incubation (44°) and the presence of bile salts thus both tend to inhibit growth of cells of *Bact. coli* into colonies, and the effects are more pronounced with cells attenuated by prolonged immersion in water.

Table 5. *Percentage of total population of* Bact. coli *able to grow on bile salts lactose agar at* 44°, *and on a similar medium without bile salts, after different periods of immersion in Ringer's solution of two different strengths*

	Ringer's solution used for immersion			
	Full-strength		¼-strength	
	Lactose agar at 44°			
Period of immersion in Ringer's solution (hr.)	With bile salts	Without bile salts	With bile salts	Without bile salts
	Counts as percentage of those on nutrient agar at 37°			
0	72·0	72·0	80·7	79·0
1	82·0	77·4	28·3	45·1
2	69·4	83·9	10·0	25·5
3	51·6	69·4	16·2	30·4
6	62·3	68·8	38·6	45·5
12	60·3	58·6	12·7	24·2
24	91·4	94·3	11·1	33·3

Differences in inhibitory action of different brands of bile salts

Two batches (N and O) of bile salts lactose agar were prepared, both being made with brands of bile salts of high quality but marketed by two different firms. A suspension of *Bact. coli* was made in 0·003 M phosphate buffer at pH 6·0, and colony counts in triplicate were determined on each of the two media at intervals over a period of 52 hr. In sixteen such tests medium O invariably yielded a higher count than medium N, the increases ranging from 25 to 100, with an average of 66 %.

Effect of 'resuscitation' on proportion of cells able to grow on bile salts lactose agar at 44°

Tests were made to see whether cells attenuated by a period of suspension in water could be so resuscitated by preliminary incubation in a nutrient medium that they were subsequently more resistant to the inhibitory action of bile salts and to a high temperature of incubation. The general principle in these experiments was to prepare the bile salts medium in two portions: (i) a broth containing the peptone and the sugar, and (ii) an agar portion containing the bile salts, neutral red and agar. Each portion contained constituents at twice the final concentration required so that mixing resulted in a medium of normal constitution. The inoculum was added to the broth, which was incubated for a period at 37° before adding to the agar portion of the medium and preparing the film in the bottle.

For preliminary resuscitation the following two sugar broths were compared (constituents as g./l.) broth E: peptone, 40; sodium chloride, 10; lactose, 10; broth F: peptone, 15; Yeastrel, 6; glucose, 10. A 45 hr. broth culture of *Bact. coli* was suspended in 0·003M-phosphate buffer at pH 6·0 (4 parts culture to 1 million parts buffer). This suspension was incubated at 20°, and at intervals samples were removed and treated as follows: 20 ml. volumes of suspension were added to 50 ml. broths E and F, and the mixtures were incubated at 37° in a water-bath. At intervals of 30 min., 3·5 ml. of each mixture were added separately to small culture bottles containing 2·5 ml. of the agar portion of the medium (consisting of 3·0 % New Zealand agar, 0·3 % bile salts 'X', and 0·02 % neutral red) and colony counts were made in the usual way.

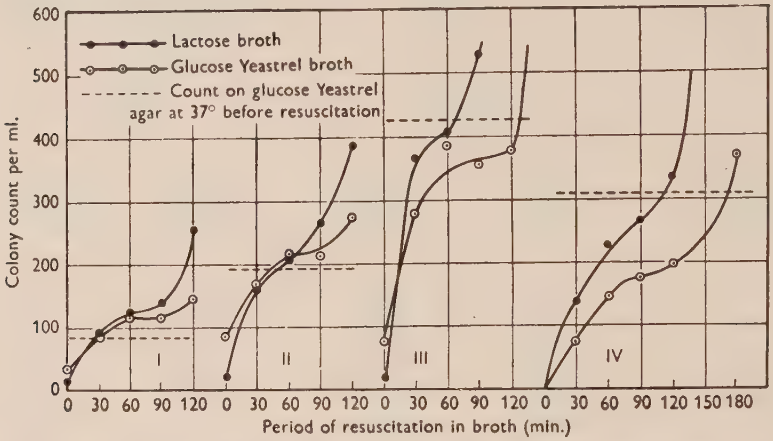

Fig. 2. Colony counts on corresponding bile salt agar medium at 45° of *Bact. coli* after period of suspension in 0·003M-phosphate buffer followed by different periods of resuscitation in (*a*) lactose broth, (*b*) glucose Yeastrel broth. (After suspension in buffer: I, for 24 hr.; II, for 48 hr.; III, for 96 hr.: IV, for 125 hr.)

Counts in the two bile salts media after different periods of resuscitation are shown in Fig. 2. In each case it appears that two effects are superimposed, resulting in a sigmoid curve. It seems likely that these two effects comprise (*a*) a preliminary period in which increasing numbers of attenuated cells become 'rejuvenated' and are able to grow on the bile salts medium, and (*b*) a period when cells which have been incubating in lactose broth or glucose Yeastrel broth come to the end of the lag period and start to proliferate. Tests showed that no appreciable growth occurred in the glucose Yeastrel broth during the first 1½–2 hr. of incubation. The two effects—resuscitation and proliferation—are, however, more distinctly separated in the glucose Yeastrel medium than in the lactose medium, suggesting that in the latter growth of some cells takes place at a somewhat earlier stage, and before all the cells have been resuscitated.

It appears from Fig. 2 that, for organisms immersed in dilute buffer for periods up to 96 hr., a count approximating to the count on glucose Yeastrel agar at 37° may be obtained by incubating the organisms for 1 hr. in lactose broth before introducing the bile salt, neutral red and agar. For organisms immersed for 125 hr. the counts so obtained would be somewhat lower. The curves suggest, in fact, that with increasing periods of immersion in buffer a decreasing proportion of total cells is capable of being resuscitated before proliferation of some of the cells begins.

Effect of neutral red on count

It was found by trial that a concentration of 0·006 % neutral red in the medium was sufficient to give easily visible red colonies after incubation. This concentration was therefore adopted in subsequent work. Tests with two samples of sewage and six samples of polluted river water, in which colony counts on bile salts lactose agar containing neutral red were compared with counts on a similar medium containing no neutral red, showed that the dye had no significant effect either on the direct count or on the count following resuscitation. Similar results were obtained with a mixture of five strains of *Bact. coli* suspended in dilute buffer solution and incubated at 20° in the laboratory. With one of these strains, tested singly, however, there proved to be a marked effect when the culture had been attenuated by a prolonged period of immersion. In the presence of neutral red the direct colony count of this strain was sometimes only a fraction of the corresponding count in the absence of the dye. The process of resuscitation appeared to remove this sensitiveness for in the subsequent colony counts, the presence of neutral red made no significant difference.

RESULTS OF APPLYING METHODS TO SAMPLES OF RIVER WATER AND SEWAGE

Direct colony counts and counts after preliminary resuscitation were determined (by the methods given in the Appendix) on sixty-five samples of polluted river water and sixteen samples of settled sewage. The Most Probable Numbers (M.P.N.) of *Bact. coli* were determined on the same samples by the usual dilution technique (primary incubation in bile salts lactose broth at 37° being followed by subculture of positive tubes into a similar medium at 44°).

Analysis of the results for river water showed that the direct colony counts (average of duplicates) ranged from 9 to 402 % with an average of 90 % of the M.P.N. Colony counts after preliminary resuscitation ranged from 37 to 676 % with an average of 177 % of the M.P.N. The wide range in percentages of colony counts is largely explained by the error of the M.P.N.

Results for the sixteen samples of sewage showed that direct colony counts ranged from 2·5 to 344 % with an average of 109 % of the M.P.N. After preliminary resuscitation the counts ranged from 80 to 2700 % with an average of 572 % of the M.P.N. The greatly increased count after resuscitation suggests that a large proportion of the organisms in sewage are in a moribund condition incapable of growing directly on the bile salts medium.

The increases in counts of water and sewage following resuscitation may be compared with the results of experiments with pure cultures. From Tables 4 and 5 it appears that if, after immersion in water, all cells could have been successfully resuscitated, the counts on bile salts lactose agar at 44° would have been increased by amounts ranging from 118 to 1233 %. In the experiments referred to in Fig. 2, resuscitation increased the counts of cells immersed for 24–96 hr. by 900–2040 %; none of the cells immersed for 125 hr. was capable of growth on the bile salts medium at 44° without previous resuscitation.

Specificity of colony count for faecal coli

Fifty-four colonies (sixteen from 'direct' counts and thirty-eight from counts after resuscitation) were isolated from fourteen samples of water and two samples of sewage, and the resulting cultures were submitted, without purification, to the indol, MR, VP, and citrate tests. Forty-seven strains gave the correct reactions for *Bact. coli* type I, six further strains conformed to type except for slight growth in citrate medium, and one strain appeared to be a 44°-positive variant of *Bact. aerogenes*. The colony counts therefore appeared to be reasonably specific for faecal coli.

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APPENDIX

Methods of making colony counts

Direct colony counts

(1) Agar medium: peptone, 20 g.; NaCl, 5 g.; bile salts,* 1·5 g.; New Zealand agar, 15 g.; distilled water, 900 ml.; pH value 7·0. (2) Lactose neutral red solution: lactose, 5 g.; 0·6 % neutral red, 10 ml.; distilled water, 90 ml. Sterilization by autoclaving at 15 lb. for 20 min.

Just before use 10 ml. of solution 2 are added to 90 ml. of the melted agar medium, and the mixture is maintained at about 48°. This is distributed in the culture bottles in quantities of 5 ml. and the bottles are maintained at the same temperature for a short time before adding the inoculum,† which consists of 1 ml. of a suitable dilution of the sample being examined. The bottles are rotated until the medium has set and are then incubated in a water-bath at 44° for 24 hr.

Colony counts after preliminary resuscitation

(1) Double-strength broth: peptone, 40 g.; NaCl, 10 g.; distilled water, 900 ml.; pH value, 7·0. Fill into bottles in 90 ml. quantities and just before use add 10 ml. of 10 % lactose solution which has been separately sterilized. (2) Double-strength agar medium: bile salts, 3·0 g.; New Zealand agar, 30 g.;

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[*] The concentration of bile salts required depends on the purity and strength of the commercial article. Guidance can usually be obtained from the manufacturers.

[†] In larger bottles inocula up to 5 ml. were used. Quantities of agar medium and of lactose neutral red solution were increased proportionately.

distilled water, 1 l.; pH value, 7·0. Fill into bottles in 90 ml. quantities and just before use add 10 ml. of 0·12 % neutral red solution which has been separately sterilized.

Solution 1 is dispensed in 7·5 ml. quantities in 6 × $\frac{3}{4}$ in. test-tubes which are incubated in a water-bath at 37°. Inocula of 3 ml. of suitable dilutions of the sample being tested are added to separate tubes and the mixtures are allowed to remain at 37° for 1 hr. Quantities of 3·5 ml. of this mixture (equivalent to 1 ml. of inoculum*) are then added to the small culture bottles to which 2·5 ml. of the agar medium have been added several minutes previously and which have been maintained at 48–50°. After rotating in the usual way the bottles are incubated in the 44° water-bath for 24 hr.

REFERENCES

Allen, L. A., Brooks, E. & Williams, I. L. (1949). Effect oı treatment at the sewage works on the numbers and types of bacteria in sewage. *J. Hyg., Camb.* **47**, 303.

Batty-Smith, C. G. (1942). The Eijkman test for faecal *coli* in the bacteriological examination of water supplies. *J. Hyg., Camb.* **42**, 55.

Clegg, L. F. L. & Sherwood, H. P. (1939). Incubation at 44° C. as a test for faecal *coli*. *J. Hyg., Camb.* **39**, 361.

Clegg, L. F. L. & Sherwood, H. P. (1947). The bacteriological examination of molluscan shellfish. *J. Hyg., Camb.* **45**, 504.

Hajna, A. A. & Perry, C. A. (1943). Comparative study of presumptive and confirmative media for bacteria of the coliform group and for faecal streptococci. *Amer. J. publ. Hlth*, **33**, 550.

Halvorson, H. O. & Ziegler, N. R. (1933). Application of statistics to problems in bacteriology. III. A consideration of the accuracy of dilution data obtained by using several dilutions. *J. Bact.* **26**, 559.

Leifson, E. (1935). New culture media based on sodium deoxycholate for the isolation of intestinal pathogens and for the enumeration of colon bacilli in milk and water. *J. Path. Bact.* **40**, 581.

Ministry of Health (1939). The bacteriological examination of water supplies. *Rep. publ. Hlth med. Subj., Lond.*, no. 71. London: H.M. Stationery Office.

Proom, H. & Hemmons, L. M. (1949). The drying and preservation of bacterial cultures. *J. gen. Microbiol.* **3**, 7.

Sherwood, H. P. & Clegg, L. F. L. (1942). Further studies of incubation at 44° C. as a test for 'faecal *coli*'. *J. Hyg., Camb.* **42**, 45.

Tai, T. Y. & van Heyningen, W. E. (1950). An easy method for counting bacterial colonies in roll tubes. *Brit. J. exp. Path.* **31**, 155.

Taylor, C. B. (1945). The effect of temperature of incubation on the results of tests for differentiating species of coliform bacteria. *J. Hyg., Camb.* **44**, 109.

Wilson, G. S., Twigg, R. S., Wright, R. C., Hendry, C. B., Cowell, M. P. & Mair, I. (1935). The bacteriological grading of milk. *Spec. Rep. Ser. med. Res. Coun., Lond.* no. 206.

* In larger bottles inocula up to 5 ml. were used. Quantities of broth and of agar medium were increased proportionately.

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Buxton, A. (1952). *J. gen. Microbiol.* 7, 268–286.

On the Transference of Bacterial Antibodies from the Hen to the Chick

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SUMMARY: Agglutinins against *Salmonella pullorum* and *Salm. typhi* (strain O 901) were induced in hens by intravenous inoculation with killed suspensions and were transferred to the yolks of their eggs. This transference of antibody continued throughout a laying season of about 280 days, and the relationship between the agglutinin titres of sera and yolks was not close. The titres of yolks seldom rose above 160 even when those in the serum were 2560 and 5120, whereas the agglutinin levels of developing ovules from hens naturally infected with *Salm. pullorum* were about the same in the related sera.

The sera and egg yolks of hens vaccinated with killed *Salm. pullorum* or infected with *Salm. gallinarum* contain a non-agglutinating antibody. The titres of non-agglutinating antibody in sera and in related egg yolks were about equal when hens were inoculated intravenously; very little antibody was demonstrable in fresh egg yolks after intramuscular or subcutaneous administration. Hens infected with *Salm. gallinarum* by the mouth yielded high yolk titres of agglutinating and non-agglutinating antibodies and these titres were maintained at a high level throughout the experimental period of 474 days. The results suggest that some of the antibody in egg yolk is formed in the follicular epithelium of the developing ovule or in the yolk material itself.

A passive immunity to *Salm. pullorum* is transferred from vaccinated hens to their chicks by the egg yolks and antibodies pass from yolk to embryonic serum from about the 11th day of incubation. The titres in the serum of the newly hatched chick are about the same as in the egg yolk before incubation and they decrease rapidly during the first 3 days and have almost disappeared by the 17th day; from the 8th day until the 17th day non-agglutinating antibodies alone are demonstrable. Antibodies are absorbed from the yolk through the vitelline and hepatic portal circulations and little or none is transferred by the yolk stalk and the chick's intestine.

Tetanus antitoxin (Ramon, 1928), diphtheria antitoxin (Jukes, Fraser & Orr, 1934; Fraser, Jukes, Branion & Halpern, 1934), neutralizing antibodies to Newcastle disease virus (Brandly, Moses, Jungherr, Jones & Tizzer, 1946) antibodies to fowl plague (Schmidt, Ørskov & Steinberg, 1936) and antibodies to the Rous sarcoma (Andrewes, 1939) may pass from the hen or duck to the egg. Agglutinins against *Salmonella pullorum* may be present in yolk from eggs of infected hens, but no comparison has been made between their concentration in hens' sera and egg yolks. Grasset (1929) could not detect any transference of passive immunity to the embryo after vaccination of hens with diphtheria toxin, and Garinei (1945) reported that antibodies against pseudo-fowl plague were not passed from the hen to the egg and chick. The transference of antibodies against Newcastle disease, fowl plague (Brandly *et al.* 1946; Levine & Fabricant, 1950) and infectious bronchitis (Jungherr & Terrell, 1948; Hofstad & Kenzy, 1950) has been observed.

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The observations herewith reported are concerned with the conditions under which agglutinating and non-agglutinating antibodies against *Salm. pullorum*, *Salm. gallinarum* and *Salm. typhi* (strain O 901) are transferred from the hen to the egg and their passage from the egg to the developing embryo.

MATERIALS AND METHODS

Pure Rhode Island Red pullets and Barred Rock pullets kept on grass were used for the vaccination experiments.

The suspensions of *Salm. pullorum* and *Salm. typhi* (strain O 901) were prepared by adding 150 ml. ethanol to 50 ml. saline washings of a 48 hr. growth; this was shaken twice daily for 2 days and stored at room temperature and a small quantity of the deposit was resuspended in saline as required.

Intravenous inoculations were made into the brachial vein, intramuscular inoculations into the thigh muscles and subcutaneous inoculations under the skin of the breast muscles; the quantities were 0·2 ml. of a suspension containing approximately 2×10^9 bacteria/ml.

The egg yolk for agglutination tests was withdrawn with a syringe after the egg shell and membranes were removed from over the air-space and the albumen discarded; 1 ml. was added to 4·0 ml. of 8·0 % (w/v) saline and the mixture was shaken and stood for 24 hr. Serial twofold dilutions of the transparent fluid below the fat were made in 8·0 % (w/v) saline and to 0·3 ml. of each dilution was added 0·3 ml. bacterial suspension. This gave a final concentration of approximately 4·4 % (w/v) saline which kept the yolk proteins in suspension.

Livetin was prepared by mixing 10 ml. of egg yolk with 30 ml. ether; this was shaken and after a few minutes the ether was withdrawn. This extraction was repeated 5 times, finally the ether was removed by centrifuging. To the egg yolk was added 36 ml. 0·85 % (w/v) saline and the mixture stored at 5° overnight. After centrifugation at 4000 r.p.m. for 30 min. an equal volume of saturated ammonium sulphate was added to the supernatant. The livetin precipitated overnight at 5° and was concentrated by centrifugation at 4000 r.p.m. for 20 min. This precipitate was resuspended in saline to give a volume of 20 ml., and after further precipitation by half saturation with ammonium sulphate it was suspended in 0·85 % saline. Any residual ammonium sulphate was removed by dialysis and the final volume of livetin made up to 24 ml.

Anti-livetin serum was prepared in rabbits by giving a series of intravenous inoculations of livetin; precipitin titres of 1/10,000 were obtained and the sera were stored at 5° without preservative.

Non-agglutinating antibodies were detected as follows: after serum samples had been tested for agglutinins, the tubes in which agglutination had not occurred were centrifuged for 15 min. at 4000 r.p.m.; the supernatant fluid was discarded and the antigen washed twice in saline. This was resuspended to its original volume in saline and an equal volume of anti-livetin rabbit serum diluted 1/40 was added. The tubes were incubated overnight at 52°. A similar technique was used for the detection of non-agglutinating antibodies in yolk, except that yolk-sensitized suspensions of antigen were washed with 8 % (w/v) saline.

Blood samples were collected from developing embryos by a method similar to that described by Beveridge & Burnet (1946) for intravenous inoculation. At least 0·2 ml. of blood was withdrawn from each egg and cellulose tape was applied over the area; the egg was then replaced in the incubator. Considerable haemorrhage sometimes occurred after the removal of the needle and the embryos died. In many other cases development continued until the 21st day but the embryos adhered to the shell membrane and failed to hatch. Blood samples were taken in this way from the 11th to the 17th days of incubation but after the 18th day the blood vessels were not easily seen by candling because of the size of the embryo. Moreover, the partial collapse of the blood vessels in the chorio-allantoic membrane made it difficult to withdraw sufficient blood.

Yolk samples were taken with a Pasteur pipette through an area of the chorio-allantoic membrane devoid of large blood vessels. Cellulose tape was applied over the areas of shell that had been removed and the eggs were returned to the incubator and kept with the perforated areas uppermost. There was a danger of a fatal leakage of allantoic fluid if the eggs were turned. There was a high mortality among embryos which had had yolk samples taken and only in a small number was it possible to take additional samples of sera and yolks at later stages. During the latter part of the work fertile eggs were incubated and removed from the incubator in groups at different stages of development; samples of blood and yolk were taken and the embryos sacrificed.

Blood samples of 0·25–0·5 ml. were taken by cardiac puncture from chicks during the first 14 days after hatching, using an all-glass syringe with a 27 gauge ½ in. needle. Chicks of 14 days of age or older were bled from the brachial vein.

A drop technique was employed with small quantities of serum and the dilutions were made directly into the agglutination tubes; the first dilution was 1/10 and incubation was at 52° in a water-bath.

RESULTS

The Passage of Agglutinins from the Hen to the Egg

Agglutinins in the sera and developing ovules of hens naturally infected with Salm. pullorum

This experiment was designed to confirm that yolk material from birds naturally infected with *Salm. pullorum* contain agglutinins (Frank & Edgington, 1937; Stoilowa, 1938), and to compare the titres in the developing ovules and sera.

Samples of serum were taken from four laying hens which were positive to the pullorum test and were from a flock in which pullorum disease existed. They were slaughtered and four or five of the largest ovules removed. The sera and yolk material from the ovules were tested for agglutinins and cultures for *Salm. pullorum*, using sodium selenite medium and deoxycholate citrate agar, were made from ovarian tissue and yolk material. The results are given in Table 1, which shows that the agglutinin titres of the individual ovules, including one known to be infected, were nearly the same as the related sera. The infected ovule showed the typical degeneration associated with chronic ovarian infection with *Salm. pullorum.*

Table 1. *Agglutinin titres of sera and ovules against* Salm. pullorum *and the results of cultural examination*

No. of hen ...	1971	4890	1926	4724
Serum	650	750	650	650
01	650*	500*	500*	325*
02	650*	650*	500*	500*
03	650*	325*	500*	500*
04	650*	500*	500†	500*
05	500‡§	—	—	—
OM	§	§	*	*

01, 02 = individual ovules.
OM = ovarian material after serological and cultural examinations had been made.
* *Salm. pullorum* not isolated.
† Yolk material from formed egg in oviduct.
‡ Ovule showing typical *Salm. pullorum* degeneration.
§ *Salm. pullorum* isolated.

Agglutinins in the sera and yolks of eggs of birds inoculated intravenously with killed Salm. pullorum

This next experiment was to observe whether results similar to the previous experiment would be obtained from hens inoculated with killed *Salm. pullorum*, and whether the transfer of antibodies from the hen to the egg continued throughout the whole laying season.

Six young Rhode Island Red hens (nos. 1498, 1499, 1500, 1501, 1502 and 1503) were vaccinated and four were kept as controls. Further doses of vaccine were given at intervals; the hens were bled periodically and their sera and egg yolks tested for agglutinins. The wide variation in the titres of egg yolks is shown in Figs. 1 and 2. With the exception of hen no. 1499 (Fig. 2) the initial serum titres were much higher than the corresponding yolk titres; this was marked in hen no. 1503 where the initial serum titre was just above 10,240. Hen no. 1500 (Fig. 1) laid eight eggs during the first 60 days of the experiment, agglutinins were not demonstrable in any of the yolks, although the serum titres varied from about 1/80 to 1/320 during this time. With few exceptions yolk titres were never above 1/160, and in most cases there was considerable variation between consecutive yolks, particularly during periods of vaccination. All tests on the sera and yolks from control birds were negative.

Agglutinins in the sera and yolks of eggs of birds inoculated intravenously with killed Salm. typhi (*strain* O 901)

A similar experiment to the one just described was made using *Salm. typhi* (strain O 901) in place of *Salm. pullorum* as a vaccine. Though there is a close antigenic relationship between these organisms, a suspension of *Salm. typhi* was found to be more sensitive to agglutinins against *Salm. pullorum* in hen sera than a suspension of the homologous bacterium. Suspensions of each organism were used in the tests made.

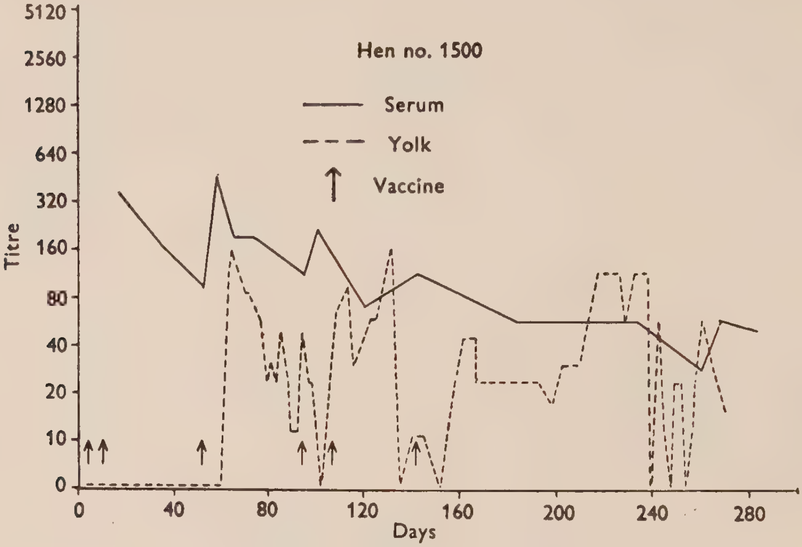

Fig. 1

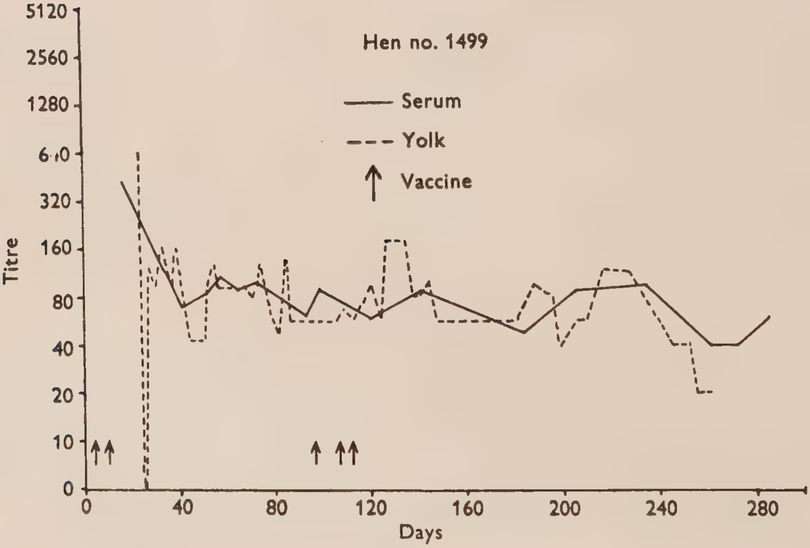

Fig. 2

Figs. 1–2. Agglutinins against *Salm. pullorum* in the sera and yolks of eggs from hens after intravenous inoculation with killed *Salm. pullorum.*

Six Rhode Island Red hens (nos. 1545, 1546, 1547, 1548, 1549 and 1550) were inoculated intravenously at intervals with killed *Salm. typhi* (strain O 901); four unvaccinated hens were kept as controls. One hen (no. 1548) died from lymphomatosis, and hen no. 1549 laid only a few eggs during the first weeks of the experiment. The results obtained from two of the hens (nos. 1546 and 1550) are shown in Figs. 3 and 4. In all cases the titres of the yolks against the typhoid suspension were higher than against the pullorum suspension and this was most apparent in hen no. 1546 (Fig. 3). The serum titres showed a greater similarity to each other than the corresponding yolk titres, and those against

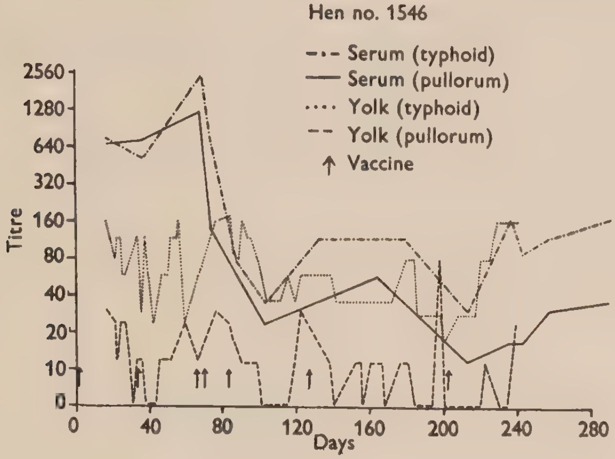

Fig. 3

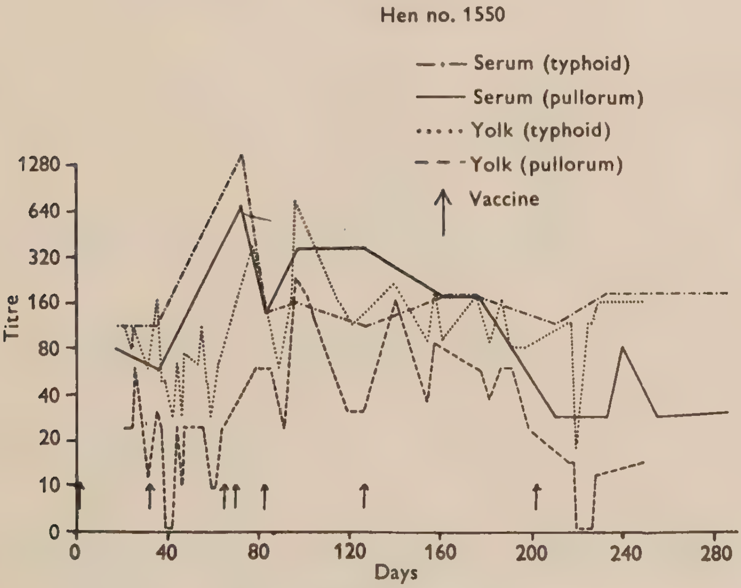

Fig. 4

Figs. 3–4. Agglutinins against *Salm. pullorum* and *Salm. typhi* in the sera and yolks of eggs from hens after intravenous inoculation with killed *Salm. typhi* (strain O 901).

the typhoid bacillus were higher. This was reversed in hen no. 1550 during the period of 80–160 days when the titre against *Salm. pullorum* was double that against *Salm. typhi.* Although there was no comparable reversion in egg yolk titres they did approximate more closely to each other in this bird during that period.

THE PASSAGE OF NON-AGGLUTINATING ANTIBODIES FROM THE HEN TO THE EGG

Non-agglutinating antibodies in the sera and yolks of eggs of hens inoculated intravenously with killed Salm. pullorum

Four Barred Rock hens (nos. 1000, 1003, 1004 and 1006) were inoculated intravenously with killed *Salm. pullorum* on the 1st and 8th days of the experiment. Samples of serum and egg yolk were tested for agglutinating and non-agglutinating antibodies, and the results from one bird (no. 1003) are given in Fig. 5.

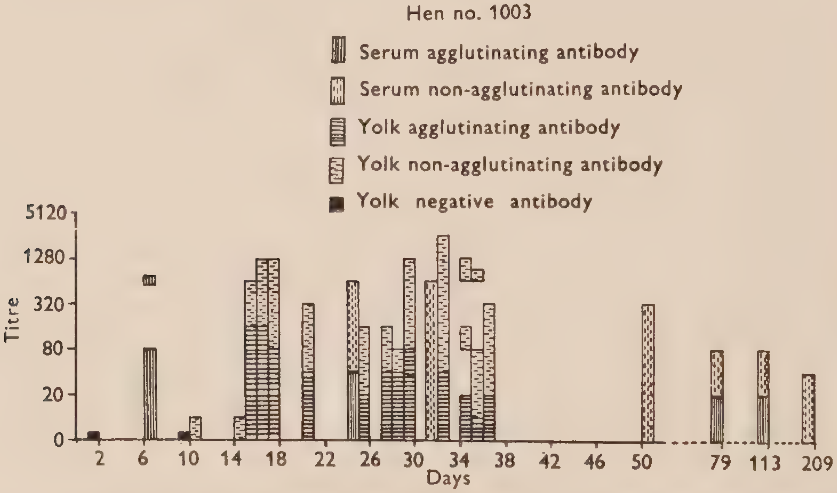

Fig. 5. Agglutinating and non-agglutinating antibodies in the serum and eggs from a hen after intravenous inoculation with killed *Salm. pullorum.*

Antibody tests on serum from hen no. 1004 are given in Table 2 for comparison with hen no. 1003. The serum titres of the four hens were rarely below 1/160; with the exception of hen no. 1003 which was 1/80 and 1/40 from the 79th to the 209th day.

Serum agglutinins were demonstrable by the 7th day and non-agglutinating antibodies in higher titres than agglutinins were observable on the 25th day, and were present in all samples up to the 209th day. No agglutinins occurred in the sera of hen no. 1003, when non-agglutinating antibody titres of 1/640, 1/320 and 1/40 were demonstrated on the 32nd, 51st and 209th days, respectively.

The earliest time at which antibodies were detected in egg yolk varied; non-agglutinating antibodies alone were detected in yolks laid by hen no. 1006 on the 6th and 8th days after the first inoculation. It was 15 and 16 days respectively, before egg yolks from birds 1000 and 1003 contained antibody titres approximating to those in their sera. The average titre of non-agglutinating antibody in egg yolks remained fairly constant, and showed a close relation to similar titres in serum.

Agglutinin titres in egg yolks varied and were lower than corresponding serum titres; in contrast to serum samples, non-agglutinating antibodies in yolks were demonstrable before agglutinins.

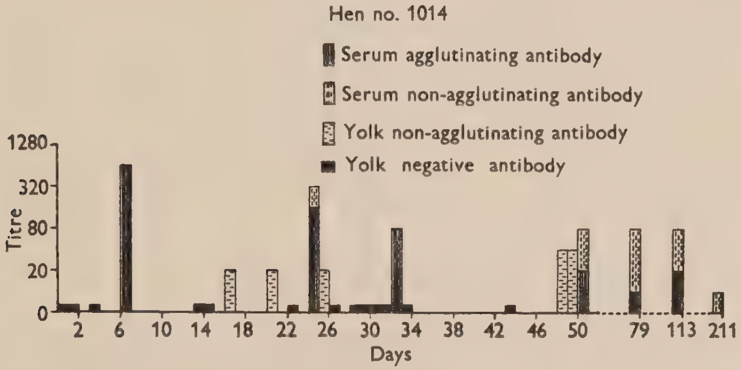

Fig. 6. Agglutinating and non-agglutinating antibodies in the serum and egg yolks from a hen after subcutaneous inoculation with killed *Salm. pullorum*.

The non-agglutinating antibodies from the two eggs laid by hen no. 1003 on the 35th and 36th days showed a zone effect; this phenomenon occurred only with these eggs and with a sample of serum from the same hen, taken on the 7th day.

Non-agglutinating antibodies in the sera and yolks of eggs from hens inoculated subcutaneously with killed Salm. pullorum

Four Barred Rock hens (nos. 1002, 1007, 1014 and 1020) were inoculated subcutaneously with killed *Salm. pullorum* on the 1st and 8th days of the experiment. The sera and egg yolks from these birds were tested for agglutinating and non-agglutinating antibodies, and the results from one bird (no. 1014) are shown in Fig. 6.

The antibody content of the sera varied particularly during the latter half of the experiment. Two hens (nos. 1002 and 1007) maintained fairly constant serum titres throughout the whole period and were in contrast to hen no. 1014 and particularly hen no. 1020. The first samples taken from all the birds on the 7th and 8th days contained agglutinins only and were similar to those from the birds which had been inoculated intravenously. The non-agglutinating antibodies were demonstrable at variable times. In hens nos. 1007 and 1014 agglutinins predominated for the first 30 days, whereas from the 50th to the 80th days onwards the non-agglutinating antibodies were in excess. Non-agglutinating antibodies appeared earlier in samples from hens nos. 1002 and 1020. They were present in all samples from the 25th day except that from no. 1020 on the 113th day; this bird showed the greatest variation in the amount and type of antibody in its serum.

The eggs from these birds were tested for antibody content, and a small amount only was transferred from the hen to the egg yolk. A yolk titre of 1/20 was the highest recorded, although the serum titres at the time most of these eggs were laid varied from 1/80 to 1/1280. The type of antibody in the egg yolks was not constant and bore no relationship to the type of antibody in the related sera.

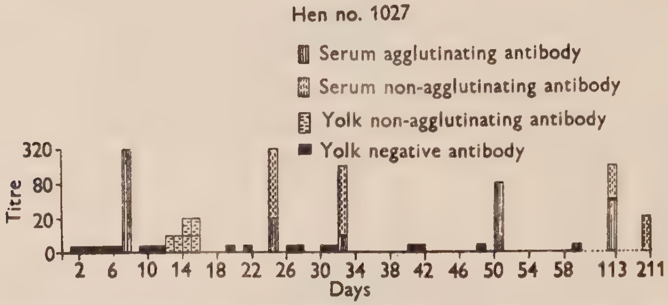

Fig. 7. Agglutinating and non-agglutinating antibodies in the serum and egg yolks from a hen after intramuscular inoculation with killed *Salm. pullorum.*

Non-agglutinating antibodies in the sera and yolks of eggs from hens inoculated intramuscularly with killed Salm. pullorum

Four Barred Rock hens (nos. 1022, 1027, 1038 and 1039) were inoculated intramuscularly with a suspension of killed *Salm. pullorum* on the 1st and 8th days of the experiment. The results of antibody tests on the sera and yolks of eggs from hen no. 1027 are shown in Fig. 7. Hen no. 1039 laid only a few eggs during the first days of the experiment and it was not possible to compare its serum and yolk antibodies; the results of the antibody tests on the serum from this bird are included in Table 2 for comparison with hen no. 1027 (Fig. 7).

The agglutinating and non-agglutinating antibodies in the sera of all these birds were similar to those in hens inoculated subcutaneously. Serum titres were maintained at a level of at least 1/40 during the first 113 days of the experiment. On the 7th day agglutinins only were present in all four sera, and this is comparable to the results obtained from the subcutaneously and intravenously inoculated groups.

The tests on egg yolks showed that only a little antibody was transferred from the hen to the yolk, and this was of the non-agglutinating kind; these results are similar to those obtained from subcutaneously inoculated hens.

Non-agglutinating antibodies in the sera and yolks from eggs of hens infected orally with living Salm. gallinarum

The purpose of this experiment was to observe the development of agglutinating and non-agglutinating antibodies in the sera of hens infected with live organisms, and to compare the results with those obtained from hens after inoculation with dead organisms by the intravenous, subcutaneous and intramuscular routes.

Four Brown Leghorn hens (nos. 1280, 1285, 1322 and 1324) aged 4 months were infected orally with 0·2 ml. of an 18 hr. broth culture of *Salm. gallinarum*. Serum samples were taken at intervals and tested for agglutinating and non-agglutinating antibodies. Two hens (nos. 1322 and 1324) died after about 50 and 110 days respectively, from the infection with *Salm. gallinarum*. The results of the antibody tests on the sera from these two birds are shown in Table 2. Seven days after infection their sera showed very low non-agglutinating antibody titres but both types of antibody were demonstrable on the 20th day. This early development of non-agglutinating antibody in hens after infection contrasts with the results obtained from inoculation of dead bacteria by the intravenous, subcutaneous and intramuscular routes. In these cases, agglutinins were the first antibodies to be detected in the sera.

Table 2. *The agglutinating and non-agglutinating antibody titres of sera and egg yolks from immunized hens*

Days of experiment	Hen no. 1004*		Hen no. 1039†		Hen no. 1324‡		Hen no. 1322‡	
	Serum		Serum		Serum		Serum	
	A	NA	A	NA	A	NA	A	NA
7	320	> 320	320	> 320	0	10	0	10
20	—	—	—	—	40	80	320	640
32	1,280	5,120	0	320	—	—	—	Died
49	—	—	—	—	2,560	5,120	—	—
113	40	640	20	80	—	Died	—	—
265	—	—	—	—	—	—	—	—
347	—	—	—	—	—	—	—	—
374	—	—	—	—	—	—	—	—
403	—	—	—	—	—	—	—	—
413	—	—	—	—	—	—	—	—
474	—	—	—	—	—	—	—	—

Days of experiment	Hen no. 1280‡				Hen no. 1285‡			
	Serum		Yolk		Serum		Yolk	
	A	NA	A	NA	A	NA	A	NA
7	80	> 80	—	—	20	80	—	—
20	5,120	20,480	—	—	320	5,120	—	—
32	—	—	—	—	—	—	—	—
49	640	2,560	—	—	2,560	5,120	—	—
113	—	—	—	—	—	—	—	—
265	640	10,240	—	—	160	2,560	—	—
347	1,280	10,240	20,480	81,920	1,280	5,120	1,280	5,120
374	—	—	10,240	163,840	—	—	—	—
403	2,560	10,240	10,240	163,840	—	—	—	—
413	—	—	—	—	—	—	1,280	20,480
474	10,240	81,920	—	—	2,560	20,480	—	—

* Intravenous inoculation killed suspension *Salm. pullorum*.
† Intramuscular inoculation killed suspension *Salm. pullorum*.
‡ Oral infection with living *Salm. gallinarum*.
A = agglutinin; NA = non-agglutinating antibody.

Two of the infected hens (nos. 1280 and 1285) survived the infection with *Salm. gallinarum* and the antibody content of their sera was tested during the period of 474 days. During the latter half of the experiment both birds came into lay and the yolks of their eggs were also tested for antibodies. These results are summarized in Table 2. Serum titres were maintained at a high level throughout the period and the non-agglutinating antibody titre reached its highest level in each bird on the 474th day after infection. The agglutinating and non-agglutinating antibody titres were as high in egg yolks as the serum titres in hen no. 1285, and in hen no. 1280 they were of ten more than double the serum titres.

THE TRANSFERENCE OF ANTIBODIES FROM THE EGG TO THE CHICK

The transference of antibodies from the yolk to the embryo

Fertile eggs were incubated, and samples of yolk and embryo serum taken at intervals between the 11th and 17th days of incubation. All samples were tested for agglutinating and non-agglutinating antibodies.

The results are given in Table 3 and show a variation in the rate at which antibodies are transferred from the egg yolk to the serum of the embryo

Table 3. *The antibody titres of yolks and related embryonic sera in eggs at different periods of incubation*

Egg no.	Day of incubation	Yolk titre		Serum titre	
		A	NA	A	NA
1	11	10	80	0	0
2	11	10	80	0	10
3	11	20	80	0	10
4	11	640	1280	0	10
5	11	80	320	0	10
6	11	1280	5120	0	10
7	11	160	320	0	160
8	11	nt	nt	0	80
9	11	nt	nt	0	20
10	12	160	320	0	20
11	12	40	40	0	20
12	14	320	2560	0	40
13	14	80	320	0	10
14	14	320	2560	0	40
15	15	40	80	0	5
16	15	20	160	5	20
17	15	40	320	0	10
18	15	40	80	1280	1280
19	17	1280	1280	40	320
20	17	80	5120	0	5
21	17	80	160	0	0
22	17	80	640	0	1280
23	17	40	80	10	20
24	11	320	640	160	160
	17			320	640
25	11	320	640	80	80
	17			0	80

A = agglutinating antibody; NA = non-agglutinating antibody; nt = not tested.

Agglutinins were never demonstrated in embryo sera from the 11th to the 14th days of incubation, and were often absent in eggs examined from the 15th to the 17th day. In most samples the presence of non-agglutinating antibodies alone was the first indication of the transference of immunity from the egg to the embryo.

The transference of antibodies from the yolk to the young chick

(1) *First experiment.* The eggs used were laid by three hens (nos. 1000, 1003 and 1006) which had been used in a previous experiment, and were collected 100–150 days after the hens had been inoculated intravenously with killed *Salm. pullorum.*

Table 4. *Antibody titres in the sera of young chicks hatched from eggs of immune birds*

No. of parent bird	Parent serum		Parent yolk		No. of chick	Age of chick (days)	Chick serum (NA)
	A	NA	A	NA			
1000	320	1,280	20	320	1	1	40
					2	1	20
1003	—	—	—	—	3	1	20
					4	1	10
1006	—	—	—	—	5	1	10
1000	—	—	—	—	6	8	20
1003	—	—	10	80	7	8	20
					8	8	10
1006	—	—	—	—	9	8	20
					10	8	20
					11	8	20
					12	8	10
1000	2,560	20,480	40	1,280	13	13	80
					14	13	80
					15	13	40
					16	13	40
					17	13	20
1003	40	320	—	—	18	13	40
					19	13	20
					20	13	10
1006	80	160	320	2,560	21	13	80
					22	13	40
					23	13	20

A = agglutinating antibody; NA = non-agglutinating antibody.

Samples of serum were taken from groups of the chicks on the 1st, 8th or 13th day after hatching and were tested for agglutinating and non-agglutinating antibodies; the results are shown in Table 4. Non-agglutinating antibody alone was observed in the sera of all the twenty-three chicks. The absence of agglutinins in these sera may be partly accounted for by the low agglutinin titres of egg yolks indicated by the antibody levels in related eggs. In the case of chicks from hen no. 1006, which were tested at 13 days of age, agglutinins may have been present in the sera soon after hatching and may have disappeared by the 13th day. Yolk titres related to chicks tested on the 1st and 8th days after hatching were lower than yolk titres related to the 13-day-old chicks, which may have been responsible for the little variation in non-agglutinating antibody titres in the sera from different ages of chicks.

(2) *Second experiment.* A group of hens recently inoculated intravenously with killed *Salm. pullorum* was used for the production of eggs; chicks were hatched from these eggs and blood samples were taken at intervals. In a few cases two samples were taken from individual chicks when they were between 1 and 17 days of age; in others, not more than one sample was taken from each chick. The results are given in Table 5 and show that in contrast to the first experiment agglutinating as well as non-agglutinating antibodies were present in the sera of the young chicks. The relationship between the titres of chick sera and of related egg yolks was not close. Chick nos. 2 and 3 had low serum titres, while egg yolk titres of the related hen (no. 918) were high. In the case of chick no. 4 the non-agglutinating antibody serum titre was 4 times as high as that in the related yolk. The sera from some chicks which were tested on two occasions showed a rapid fall in the level of antibodies from the 1st to the 3rd days after hatching, and this continued until the 14th to 17th days when the results were negative.

Table 5. *Antibody titres in the sera of young chicks hatched from eggs of immune birds*

No. of parent bird	Parent serum		Parent yolk		No. of chick	Age of chick (days)	Chick serum	
	A	NA	A	NA			A	NA
918	2,560	>2,560	1,280	5,120	1	1	320	>320
	2,560	>2,560	2,560	>2,560	2	1	80	>80
	2,560	>2,560	2,560	>2,560	3	1	20	nt
919	320	320	320	2,560	4	1	2,560	10,240
923	1,280	2,560	1,280	2,560	5	1	640	2,560
913	—	—	—	—	6	3	5	40
914	1,280	2,560	320	1,280	7	1	1,280	10,240
						3	80	320
924	20	80	20	40	8	3	0	40
						17	0	0
	20	80	20	40	9	3	5	80
						17	0	10
916	320	640	80	320	10	3	80	160
						17	0	160
	320	640	80	320	11	7	5	20
						14	0	10

A = agglutinin; NA = non-agglutinating antibody; nt = not tested.

The experiments on the transference of antibodies from the egg to the embryo and chick are summarized in Fig. 8. This shows the rise of antibody level in embryo serum from the 11th day to the end of incubation. The maximum serum titre appeared on the 1st day after hatching and was slightly higher than the average yolk titre. Non-agglutinating antibody formed a large proportion of the total antibody in most of the serum samples, and in chicks from 8 to 17 days of age it was the only antibody which could be demonstrated.

Route of absorption of antibodies by the developing embryo and young chick

It has been shown that the developing embryo absorbs antibodies from the yolk sac during the latter half of the incubation period, and that the concentration in the embryo's serum reaches its maximum at about hatching time. Thereafter, the level of antibodies in the serum of the young chick, which is mostly non-agglutinating in nature, rapidly declines. This high proportion of non-agglutinating antibody which occurs when the total antibody level is being reduced may be associated with the protein metabolism of the chick, and with the route of absorption of yolk by the embryo and newly hatched chick.

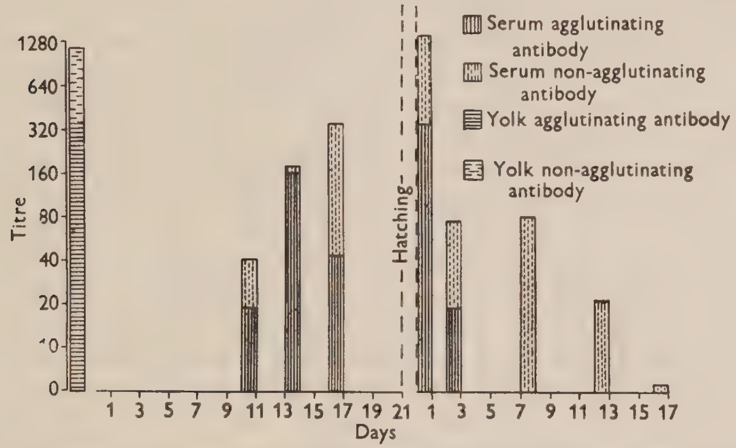

Fig. 8. The transfer of agglutinating and non-agglutinating antibodies from the egg yolk to the developing embryo and young chick.

The contents of the yolk sac are the main source of food supply for the developing embryo, and according to Lillie (1927) yolk is absorbed through the walls of the yolk sac into the vitelline circulation, and finally to the liver by way of the hepatic blood vessels. The yolk sac is connected to the duodenum by the so-called yolk stalk, and Calhoun (1933) and Kar (1947) have shown that this stalk is hollow, and may help in the absorption of yolk by the embryo, although the yolk material does not pass into the intestines of the chick. Masurier, Branion & Marcellus (1946) observed the rate of absorption of yolk material by the young chick, and reported that chicks of 5 weeks old contained unabsorbed yolk material. In some chicks the yolk sac was attached to the intestines by the stalk and in others the stalk had either disappeared or was in various stages of atrophy. In the opinion of these authors: 'It would seem that chicks have the ability to absorb the yolk regardless of whether or not the yolk stalk remains attached to the yolk sac.'

Two small experiments designed to find out whether antibodies in egg yolk were absorbed by either (*a*) the vitelline circulation, or (*b*) by the intestines via the yolk stalk or by both these routes, are described below.

(*a*) Each of six embryos was inoculated into the yolk sac on the 8th day of incubation, with 0·4 ml. of adult hen serum having an agglutinating titre of 1/640 to *Salm. gallinarum*. The eggs were then re-incubated and four of them hatched. Serum samples from these four chicks were taken 3 days after hatching and tested for agglutinins. One sample had a titre of 1/20 and the remaining three had titres of 1/10. The average size of an unincubated egg yolk is 17·1 ml. (Romanoff & Romanoff, 1949), and the inoculation of 0·4 ml. of serum into the yolk sac would give a dilution of at least 1/40 of the original serum. The dilution of this serum with an agglutinin titre of 1/640 would reduce the titre to about 1/15; this agrees closely with the results obtained. This experiment was made before the rate of the fall of antibodies in chick serum had been ascertained, and judging by Fig. 8 it is assumed that the agglutinin titres would have been higher on the first day after hatching.

(*b*) The alternative route of yolk absorption to the vitelline and hepatic blood vessels is via the intestine; accordingly, each of twelve newly hatched chicks was, on two occasions, given an oral dose of 0·4 ml. of hen serum containing antibodies against *Salm. pullorum*. Blood samples were then taken and examined for agglutinins; the data in Table 6 shows the low antibody content of the chick sera. The blood volume of the day-old chick is about 2·5 ml. and the dilution of the administered serum can be calculated. The results show that only a small proportion of available antibody protein was absorbed by the chick's intestines in a form in which it could be detected by immunological tests. It is apparent from these two experiments that most of the antibody in the egg yolk of young chicks is absorbed by the vitelline and hepatic circulation.

Table 6. *The absorption of antibodies by the young chick after oral administration of immune serum*

No. of chicks	Titre of dosing serum		Dose (oral) (ml.)	Age of chicks when dosed (hr.)	Age of chicks when tested (hr.)	Chick serum titre (average)	
	A	NA				A	NA
4	5,120	> 5,120	0·4	12, 30	36	17	40
					48	8	10
4	1,280	> 1,280	0·4	36, 48	60	4	2
4	1,280	> 1,280	0·4	60, 78	80	7	—
					96	4	—

A = agglutinating antibody; NA = non-agglutinating antibody.

DISCUSSION

The passage of antibodies from the hen to its eggs is probably not a simple process of transference of unaltered antibody molecules. The antibody level of eggs laid by hens vaccinated with killed organisms is lower than in eggs from hens naturally infected with *Salm. pullorum*, and in those vaccinated intravenously there is some alteration to the agglutinin either in its molecular structure or in its relationship to some substance in egg yolk that inhibits agglutination. The physiology of the development of the hen's ovule is not well understood, and Conrad & Scott (1938) have suggested three possible ways by which yolk might be formed. They suggested that in immunized hens, the antibodies either passed directly into the developing ovule, or that on reaching the follicular epithelial cells the globulin molecules were hydrolysed to polypeptides and perhaps peptides. In this state they were then secreted into the ovule where they became reformed into globulin molecules. The amount of globulin in egg yolk is greater (per unit volume) than in serum, and whichever method of globulin accumulation occurred in egg yolk, a greater concentration of antibodies in yolk than in serum is to be expected; this is similar to the accumulation of antibody in bovine colostrum (Marrack, 1947; McDougall, 1949; Smith & Holm, 1948).

The route of inoculation of killed bacteria into a laying hen clearly influenced the quantity of antibody subsequently demonstrable in the yolks of eggs. The intravenous route induced equivalent non-agglutinating antibody titres in yolks and related sera; in contrast, subcutaneous and intramuscular inoculations induced little demonstrable antibody in egg yolks. Experimental infection of hens with living bacteria produced relatively high titres in the egg yolks, and it appears as if infection with *Salm. gallinarum* and intravenous inoculation with killed *Salm. pullorum* enables the bacteria to come into direct contact with ovarian tissue, and the hen's ovary is a common site of infection with these bacteria. These results suggest that some of the antibody in egg yolk may have been produced by ovarian tissue, and that a small proportion only of the circulatory antibody produced in other organs was absorbed by the developing ovules.

No clear relationship between agglutinating and non-agglutinating antibodies has been observed, and their relative proportions in serum and in yolk bear no relationship to each other. There is no evidence to suggest that non-agglutinating antibodies are derived from agglutinins passing through the follicular epithelium into the developing ovule. On the contrary, the identification of agglutinins in yolk at a time when non-agglutinating antibodies alone are demonstrable in related serum, suggests that some of these agglutinins are formed from constituents of protein molecules in the follicular epithelial cells or in the developing ovules.

Jukes & Kay (1932) found that proteins form 3·1 g. of a total of 9·4 g. organic matter of hen's egg, and according to Romanoff & Romanoff (1949) 16·6 % of egg yolk consists of protein. Eagle (1932) and Follensby & Hooker (1939) have shown that an agglutination reaction may be delayed or even inhibited when proteins other than those directly associated with antigen-antibody reactions are present. It was to be expected that the high concentration of protein in egg yolks would result in relatively greater concentrations of demonstrable antibody in yolk than in the serum of immune birds, and that the proteins not associated with antigen-antibody reactions would inhibit agglutination but would not interfere with the sensitization of antigen with antibody. Under these conditions non-agglutinating antibody in egg yolks would combine with antigenic constituents similar to those which combine with agglutinins, and this similarity would be demonstrated by a suitable ‘blocking test’. In experiments not yet published, no such similarity between agglutinins and non-agglutinating antibodies has been found either in egg yolk or in serum; these confirm the findings of Morgan & Schütze (1946) who examined the properties of non-agglutinating antibodies against *Salm. typhi* and *Shigella shigae* in human sera.

Antibodies against *Salm. pullorum* transferred to the egg, increase in concentration in the sera of embryos during incubation and rapidly decrease in the sera of young chicks after hatching. Agglutinins disappear more rapidly than non-agglutinating antibodies, and these alone persisted from the 8th to the 17th days after hatching. Moore, Shen & Alexander (1945) examined the plasma of developing chick embryos and showed that during embryonic development the plasma underwent a gradual differentiation from a simple to a more complex pattern, associated with the inclusion in the plasma of various constituents of egg yolk. Schechtman & Nace (1950) and Marshall & Deutsch (1950) confirmed these results and have shown that the egg yolk constituents in the serum disappear from about the 5th day after the chick has hatched. Brandt, Clegg & Andrews (1951) examined the protein constituents of serum of male and female chicks of 4–7 weeks of age and older by electrophoretic and salt-fractionating methods. The β-globulin and albumin fractions remained unchanged during the period of observation, but the α- and γ-globulin fractions increased with age. They also identified a fast-moving component which was present in the sera of laying hens but not in the sera of young pullets or male birds.

The transfer of antibodies from mother to young in mammals occurs either during intra-uterine life or by way of the colostrum and the intestines of the newborn. In the rabbit, however, it has been shown (Brambell, Hemmings & Henderson, 1951) that the route of absorption is by the uterine lumen and the yolk-sac splanchnopleur. This passage of antibodies through the vitelline circulation of the yolk-sac splanchnopleur is comparable to the route of transfer of antibodies from the egg yolk to the developing avian embryo.

The developing avian embryo is supplied with protein and the proteolytic enzymes in the yolk and the embryo render this protein readily available. There is practically no alteration in the quantity or type of antibody transferred from the yolk to the embryo, and in the young immune chick, the digestion of certain protein fractions corresponds with a reduction in circulating antibody. Agglutinins disappear during the first few days after hatching, and from about the 8th to the 17th days non-agglutinating antibodies only are demonstrable. These conditions suggest that non-agglutinating antibodies may be more resistant to breakdown by proteolytic enzymes than agglutinins. It has been shown (Buxton, 1951) that under suitable conditions the treatment of agglutinins with a proteolytic enzyme results in the alteration of the demonstrable antibody into the non-agglutinating type.

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REFERENCES

ANDREWES, C. H. (1939). Occurrence of neutralising antibodies for Rous sarcoma virus in sera of young 'normal' chicks. *J. Path. Bact.* **48**, 225.

BEVERIDGE, W. I. B. & BURNET, F. M. (1946). The cultivation of viruses and rickettsiae in the chick embryo. *Spec. Rep. Ser. med. Res. Coun., Lond.* no. 256.

BRAMBELL, W. F. R., HEMMINGS, W. A. & HENDERSON, M. (1951). *Antibodies and Embryos.* London: Athlone Press.

BRANDLY, C. H., MOSES, H. E., JUNGHERR, E. L., JONES, E. E. & TIZZER, E. E. (1946). Newcastle disease and fowl plague investigations in the war research programme. *J. Amer. vet. med. Ass.* **108**, 369.

BRANDT, L. W., CLEGG, R. E. & ANDREWS, A. C. (1951). The effect of age and degree of maturity on the serum proteins of the chicken. *J. biol. Chem.* **191**, 105.

BUXTON, A. (1951). Transfer of bacterial antibodies from the hen to the chick. *Nature, Lond.* **168**, 657.

CALHOUN, M. L. (1933). The microscopic anatomy of the digestive tract of *Gallus domesticus*. *Iowa St. Coll. J. Sci.* **7**, 261.

CONRAD, R. M. & SCOTT, H. M. (1938). The formation of the egg of the domestic fowl. *Physiol. Rev.* **18**, 481.

EAGLE, H. (1932). Specific agglutination and precipitation. II. Velocity of reaction. *J. Immunol.* **23**, 153.

FOLLENSBY, E. M. & HOOKER, S. B. (1939). The effect of temperature upon combination and aggregation and equilibrium in reaction between antigen and antibody. *J. Immunol.* **37**, 367.

FRANK, N. A. & EDGINGTON, B. H. (1937). Agglutinin content of eggs from *Salmonella pullorum* infected hens. *Poult. Sci.* **16**, 442.

FRASER, D. T., JUKES, T. H., BRANION, H. D. & HALPERN, K. C. (1934). The inheritance of diphtheria immunity in ducks. *J. Immunol.* **26**, 437.

GARINEI, V. (1945). Lack of transmission of immunity through the egg in fowl plague. *Clin. vet., Milano*, **68**, 18.

GRASSET, E. G. (1929). Recherches sur la sensibilité du tissu embryonnaire aux antigènes. Essais d'immunisation comparée de l'embryon de poulet et de la poule adulte. *C.R. Soc. Biol., Paris*, **101**, 1102.

HOFSTAD, M. S. & KENZY, S. G. (1950). Susceptibility of chicks hatched from recovered hens to infectious bronchitis. *Cornell Vet.* **40**, 87.

JUKES, T. H., FRASER, D. T. & ORR, M. D. (1934). The transmission of diphtheria antitoxin from hen to egg. *J. Immunol.* **26**, 353.

JUKES, T. H. & KAY, H. D. (1932). Egg yolk proteins. *J. Nutrit.* **5**, 81.

JUNGHERR, E. L. & TERRELL, N. L. (1948). Naturally acquired passive immunity to infectious bronchitis in chicks. *Amer. J. vet. Res.* **9**, 201.

KAR, A. B. (1947). Fate of the yolk-stalk in the domestic fowl. *Poult. Sci.* **26**, 108.

LEVINE, P. P. & FABRICANT, J. (1950). Susceptibility to Newcastle disease infection of chicks with congenital serum antibodies. *Cornell Vet.* **40**, 213.

LILLIE, F. R. (1927). *The Development of the Egg.* New York: Henry Holt.

MACDOUGALL, E. I. (1949). An immune globulin fraction from bovine precolostrum. *Biochem. J.* **44**, 531.

MARRACK, J. R. (1947). Lactation: function and product. Antibodies in milk. *Brit. med. Bull.* **5**, 187.

MARSHALL, M. E. & DEUTSCH, H. F. (1950). Some protein changes in fluids of the developing chicken embryo. *J. biol. Chem.* **185**, 155.

MASURIER, H. E. LE, BRANION, H. D. & MARCELLUS, F. N. (1946). Observations on unabsorbed yolk in chicks. *Poult. Sci.* **25**, 300.

MOORE, D. H., SHEN, S. C. & ALEXANDER, C. S. (1945). The plasma of developing chicks and pig embryos. *Proc. Soc. exp. Biol., N.Y.* **58**, 307.

MORGAN, W. T. J. & SCHÜTZE, H. (1946). Non-agglutinating antibody in human antisera to *Sh. shiga* and *S. typhi. Brit. J. exp. Path.* **27**, 286.

RAMON, G. (1928). Sur le passage de la toxine et de l'antitoxine tétanique de la poule à l'œuf et au poussin. *C.R. Soc. Biol., Paris*, **99**, 1476.

ROMANOFF, A. L. & ROMANOFF, A. J. (1949). *The Avian Egg.* New York: J. Wiley and Sons.

SCHECHTMAN, A. M. & NACE, G. (1950). Development of serum proteins in the embryonic chick. *Anat. Rec.* **106**, 436.

SCHMIDT, S., ØRSKOV, J. & STEINBERG, E. (1936). Immunisation active contre la peste aviare. *Biol. Medd., Kbh.* **12**, 1.

SMITH, E. L. & HOLM, A. (1948). The transfer of immunity to the newborn calf from colostrum. *J. biol. Chem.* **175**, 349.

STOILOWA, E. R. (1938). Die Dotteragglutination bei der Kükenruhr. *Z. InfektKr. Haustiere*, **53**, 236.

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Brieger, E. M. & Glauert, A. M. (1952). *J. gen. Microbiol.* 7, 287–294.

A Phase-contrast Study of Reproduction in Mycelial Strains of Avian Tubercle Bacilli

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SUMMARY: The reproductive processes occurring in mycobacteria were studied in mycelial strains of avian tubercle bacilli and the developments recorded by phase-contrast microscopy. Three main developments were traced: (1) Disintegrating mycelial branches release micro-rods (as small as 0·2 μ.), which later elongate into bacillary forms. (2) Parts of some mycelia produce filamentous sprouts which grow into entangled nests of filaments, and finally give rise to spherical colonies of short rods. (3) Some mycelial branches become swollen and very dense. Later they become more transparent and parallel rows of filaments are revealed lying orientated along the length of the branches. The typical morphology of orientated and non-orientated growth is explained with reference to the modes of development. Growth is preceded by a stage of mycelial proliferation, which is mainly non-acid-fast, but results in microcolonies of the normal acid-fast rod. The relation between some atypical structures and L-forms is discussed.

In previous publications (Brieger, 1945; Brieger & Fell, 1945, 1946; Brieger & Robinow, 1947), evidence was submitted to show that the mycelia often seen in cultures of avian strains of tubercle bacilli, and usually described as branching forms, are not a mere abnormality of growth. Warm stage observation of living cultures made with the ordinary microscope showed how some of the filaments grew by budding, branching and elongation to form complex mycelial structures (Brieger & Fell, 1945). Chromatinic structures were demonstrated using the acid-Giesma stain, and their presence indicated that reproductive processes were taking place in the mycelial forms (Brieger & Robinow, 1947). However, from the results obtained the relation between the mycelial and ordinary rod-shaped forms was not altogether clear. It was uncertain whether the two types merely co-existed or whether one form could develop into the other.

In the present study the phase-contrast microscope has been used to make daily observations of living material grown in coverslip cultures. The good contrast available has enabled the relationships between the various forms to be closely examined, and has revealed structural details not seen with the ordinary microscope.

MATERIALS AND METHODS

The strain used in these experiments was recovered from the liver and spleen of a tuberculous fowl in November 1950, and was kindly supplied by the Ministry of Agriculture's Research Station, Weybridge. Subcultures were made on Löwenstein's medium as shown in Table 1.

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Table 1. *Details of subcultures on Löwenstein's medium*

No. of subculture	Date of subculture	No. of experiment	Age of culture when used (weeks)	Characteristics of forms in starting preparations
1	24. xi. 50	—	—	No mycelia, short rods
2	27. xii. 50	CG 1	5	Isolated mycelia, rods predominant
	27. xii. 50	CG 2	9	Isolated mycelia, rods predominant
3	19. ii. 51	CG 3	3	Isolated mycelia, rods predominant
	19. ii. 51	CG 4	7	Mycelia predominant
4	7. iii. 51	CG 5	7	Mycelia predominant
	7. iii. 51	CG 6	8	Mycelia predominant

Technique

Observations were made on cover-slip cultures prepared in a similar way to tissue cultures (Strangeways, 1924). Small drops of chick embryo extract and fowl plasma were mixed on $1\frac{1}{4}$ in. no. 0 cover-slips and spread into a circle. The excess fluid was immediately removed with a pipette so that a thin film of medium was formed before clotting occurred. A loopful of the culture was suspended in saline and the cover-slips inoculated with a thin film of the suspension. Each cover-slip was then inverted over a brass frame (1 mm. thick) which had previously been sealed to a thin slide and the preparation left on the bench in an inverted position for some hours. This procedure was necessary to enable the bacilli to settle on the surface of the medium: even with it the movements of the bacilli frequently made photography difficult and on some occasions the preparation had to be fixed before recordings could be made. The living cultures were incubated at 37·5° for the period of the experiment.

Observations were made with a Cooke, Troughton and Simms microscope equipped with phase-contrast condenser and objectives. A reflex camera was adapted to take photographic recordings on 35 mm. Microfile film. Each day frequent observations were made of the preparations with the $\frac{1}{12}$ in. oil-immersion objective, and photographs taken to record all the typical forms present. This was usually continued for about 3 weeks when staining showed that acid-fast microcolonies had invariably formed. At each stage parallel cultures were fixed and stained by various methods (to be described in a later publication) and the results compared.

At intervals the microscope was placed in a hot-box and a certain field was selected and viewed continuously for several days. Experience has shown that the value of such observations is limited by the fact that developments in the selected field are not necessarily typical of developments in the culture as a whole. In one field where four small mycelia were observed for 5 days it was found that three remained unchanged throughout that time, while the fourth developed rapidly and strikingly between the 3rd and 5th days of incubation, producing a mass of fine elongated filaments. This technique gives valuable information about changes in individual organisms, but in order to obtain a correct picture of all the main developments in the culture, a method would have to be adopted which would enable a far larger area to be studied continuously. As the distribution of the developing microcolonies is very irregular a study of adjacent areas would not necessarily solve the problem.

RESULTS

The results of the series of experiments can be placed in two distinct groups. In Exps. CG 1–CG 3 the development was mainly non-mycelial, while in Exps. CG 4–CG 6 the mycelial growth type predominated. The final product of the two types of development was the same: microcolonies of normal acid-fast rods.

Development of the Filamentous Type (Exps. CG1, CG 2 and CG 3)

The growth in the non-mycelial forms seems to follow the recognized type of bacterial multiplication. The inoculum consists mainly of small rod-shaped forms varying in size up to 1 μ. in length (Pl. 1, fig. 1). After 5–7 days' incubation at 37·5° the rods are no longer seen and the field is covered with long filaments of 8–10 μ. in length (Pl. 1, fig. 3). They are usually bent, vary in thickness and often have tapering or club-ends. Some are in groups and there is evidence of slight budding. Although complex mycelial structures are not present, the arrangement of the filaments is often suggestive of the mycelial form of reproduction. In fact, their method of development is very different from that of normal bacteria, and observation during the first few days of incubation shows that the final filaments are formed as a result of a germination process. The original micro-rods produce fine filamentous sprouts and in one experiment (CG 2) their appearance after 48 hr. incubation (Pl. 1, fig. 2) was similar to that seen in the streptomycetes. The sprouting filaments are bent and sometimes beaded, and possibly correspond with germination tubes.

Development of the Mycelial Type (Exps. CG 4, CG 5 and CG 6)

Starting preparation of the inoculum

In the starting preparations the picture differs from that seen in the non-mycelial cultures. Mycelial structures are now predominant, isolated rods are in the minority and many aggregates of small, dense, elements (0·5–1·0μ.) are present (Pl. 1, fig. 4). These aggregates may have resulted from a sprouting or division of small rod-shaped forms, and this may account for the great variety in grouping observed. The phase-contrast microscope also reveals the presence of minute, dense and usually spherical particles. Their dense centres are near the limit of resolution of the microscope, and they are frequently surrounded by some material of less density, which may be a form of outgrowth and gives them an irregular outline.

The mycelial structures consist of filaments with a few buds and branches. They show variations in thickness and density, and vacuoles and dense swellings occur. The various branches appear to be in different stages of development, and in some cases are apparently disintegrating.

Stage of mycelial proliferation (1–5 *days*)

During the following days of incubation rapid changes occur. The small elements increase markedly in number, become less dense and sometimes assume a rod-like form (Pl. 1, fig. 5). The irregular aggregates begin to contain increasing numbers of rods, and some clumps produce fine filamentous sprouts. Various changes occur simultaneously in the mycelia; they sprout fine filaments, their branches elongate, obvious internal structure is seen; they break up into fragments and they continue to grow on different levels. In the course of this development highly complex structures are formed which we have found to be characteristic of this phase of the mycelial type of growth (Pl. 1, fig. 6). They consist of a mass of entangled filaments and they are unlike anything described by earlier workers, mainly due to the fact that they are non-acid-fast and do not give a clear picture when viewed unstained in transmitted light. However, as will be shown in a later communication, they form the non-acid-fast matrix within which the acid-fast elements may regerminate. Forms also occur which bear some resemblance to the zooglea of Alexander-Jackson (1945) and the L-forms of Klieneberger-Nobel (1951) (Pl. 4, figs. 17, 18). In our cultures similar forms seem to have some relation to the mycelial type of reproduction.

Stage of development of microcolonies

Development of spherical colonies. The first definite stage in the development of spherical colonies of bacilli is the formation of small nests of fine filaments (Pl. 2, fig. 9). These are seen on about the 5th day of incubation and result from the proliferation of the sprouts present after 24 hr. They originate either in dense parts of the mycelia (Pl. 2, fig. 10), or in the aggregates of small organisms (Pl. 2, fig. 9). The filaments grow out in all directions and give the resulting structures a disorientated appearance. In some microcolonies the angular growth type (Ørskov, 1923) is very striking. At later stages the continued filamentous proliferation transforms the nests into small microcolonies which begin to assume a spherical shape (Pl. 2, fig. 11). Owing to their increasing thickness it now becomes difficult to focus all the various levels, and individual filaments are not easily distinguishable. The centres of the clumps acquire a granular appearance (Pl. 2, fig. 12) due to the marked beading of the component filaments. Finally, after about 18 days, these filaments separate from the periphery of the colony. Sometimes the association of the final colony with an original mycelium is still clear (Pl. 2, fig. 11). This type of microcolony formation is mainly confined to the more fluid parts of the preparations.

Development of cords. The development of cords begins after about 5 days when some mycelial branches become very dense and later give rise to parallel rows of bacillary forms (Pl. 3, fig. 13). During the following days of incubation some of these cords grow into long strands of bacilli, and after about 19 days the rods separate out and are finally released. These cord-formations predominate in the drier parts of the cultures, although they are also seen associated with the spherical colonies (Pl. 3, fig. 14). It seems likely that the two types of growth can take origin from different sections of the original mycelial complex.

Production of micro-rods. In parallel with these two types of development micro-rods are seen to develop within some of the mycelia. Other mycelia disintegrate and directly release large numbers of micro-rods containing bipolar densities (Pl. 2, fig. 7). These later elongate into denser, short filaments, so that colonies of bacilli appear. The form of the original mycelium is often still apparent (Pl. 2, fig. 8).

Structure of the final bacilli

It is remarkable that these different types of mycelial growth all result in the formation of the typical bacillary form (Pl. 4, figs. 15, 16). The bacilli vary in length up to about 2 μ. and often appear to divide by a process of simple fission. Some appear beaded in these living cultures (Pl. 4, fig. 15) and are associated with bipolar micro-rods.

From stained preparations it can be shown that the final products of all these three types of development are acid-fast, although the preceding mycelial forms are frequently non-acid-fast.

DISCUSSION

In this study phase-contrast microscopy has provided evidence of similarities between reproduction in avian strains of tubercle bacilli and in the ray fungi (Orskov, 1923). A germination process was seen to occur in the early stages of development. Germination of granular forms has also been described by Sweaney (1928), Alexander-Jackson (1945) and Nedelkovitch (1950) in human-type bacilli. Whether the fine sprouts formed are true germination tubes remains uncertain; they may be merely filamentous outgrowths from resting cells.

In 1945, Alexander-Jackson described zoogleal forms in cultures of human tubercle bacilli. In our cultures similar forms were present (Pl. 4, figs. 17, 18) and were related to mycelial fragments containing globoid bodies. Klieneberger-Nobel (1951) has referred to the possibility that L-forms occur in the reproductive cycle of tubercle bacilli, but so far we have no evidence to show that the forms we have seen develop in a way similar to L-forms.

Beading was seen in living cultures in the mycelial branches and in the final bacilli (Pl. 4, fig. 15). Their nature is uncertain but they may correspond to the chromatinic bodies described by Brieger & Robinow (1947); they cannot be mere artefacts as suggested by Porter & Yegian (1945). These beads gave rise to the rod-shaped elements which developed within the mycelial branches. Sometimes a whole section of the mycelium broke up into a mass of micro-rods (Pl. 2, figs. 7, 8). Phase-contrast observation showed them to be bipolar rods and not granules. They were sometimes as small as 0·2 μ. in length and thus comparable in size with psittacosis bodies and the larger rickettsias. If only the polar densities were detected the rods might be described as granules (Kahn, 1932), and they may easily be the elements assumed by Wyckoff (1934) to be merely short segments of rods.

In following the development of the mycelial forms we have seen how they give rise to acid-fast microcolonies of two types. These correspond closely to those described recently by Middlebrook (1950) in human bacilli. We could trace the different developments that lead to the production of the two types of microcolony. The filaments in the spherical colonies appear to be free to grow in any direction without restraint, and this results in a completely disorientated arrangement in the developing colonies. In contrast, the bacilli forming the cords appear to be constrained to lie in parallel rows, and are originally packed within dense mycelial branches. Presumably some substance similar to Bloch's 'cord factor' (1950) holds them together. The striking fact in the present observations is that the two types of colony can be seen to develop from different parts of the same mycelial structure.

The shape of the bacilli in the various stages of development differed considerably from the mature (acid-fast) rod shown in Pl. 4, fig. 15. They varied in density and in particular the bacilli at the edges of the cord-like colonies had the same 'pin-like terminal sprouts' described by Brieger & Fell (1946).

The amazing variety of forms revealed by the phase-contrast microscope when used to study living cultures of avian tubercle bacilli explains the pleomorphism first described by Maffucci (1892). By this method of daily observation it was possible to demonstrate the relation of these forms to each other and their place in the reproductive process. However, the present results do not enable us to classify the avian bacillus among the actinomycetes, streptomycetes or nocardias with which they have often been identified. In spite of certain similarities the differences are even more striking, and it may be that the development we have described is a distinguishing feature of the mycobacteria.

REFERENCES

ALEXANDER-JACKSON, E. (1945). A hitherto undemonstrated Zoogleal form of *Mycobacterium tuberculosis. Ann. N.Y. Acad. Sci.* **46**, 127.

BLOCH, H. (1950). Enzymatic characteristics of suspensions of different mycobacteria. *Amer. Rev. Tuberc.* **61**, 270.

BRIEGER, E. M. (1945). Observations on the species pathogenicity of the tubercle bacillus in intra-amniotic infection. *J. Path. Bact.* **57**, 282.

BRIEGER, E. M. & FELL, H. B. (1945). Warm-stage observations on the initial development of the avian tubercle bacillus cultivated in embryo extract. *J. Hyg., Camb.*, **44**, 158.

BRIEGER, E. M. & FELL, H. B. (1946). Warm-stage observations on the development of pseudo-mycelia in cultures of avian tubercle bacilli grown in dilute embryo extract. *J. Hyg., Camb.* **44**, 256.

BRIEGER, E. M. & ROBINOW, C. F. (1947). Demonstration of chromatinic structures in avian tubercle bacilli in the early stages of development. *J. Hyg., Camb.* **45**, 413.

KAHN, M. C. (1932). Weitere Bemerkungen zur morphologischen Untersuchung über den Entwicklungszyklus der Einzelzelle des Tuberkelbazillus. *Zbl. Bakt.* (1. Abt. Orig.), **125**, 451.

Klieneberger-Nobel, E. (1951). Filterable forms of bacteria. *Bact. Rev.* **15**, 77.

Maffucci, A. (1892). Die Hühnertuberkulose. *Z. Hyg. InfektKr.* **11**, 445.

Middlebrook, G. (1950). Recent advances in bacteriological research in tuberculosis. *Recent Adv. Tuberc. Res.* **3**, 132.

Nedelkovitch, J. (1950). Mode de multiplication du bacilli de Koch. Morphologie du bacilli et de ses colonies. Quelques sources d'erreurs. *Ann. Inst. Pasteur*, **78**, 177.

Orskov, J. (1923). *Investigations into the Morphology of the Ray Fungi.* Copenhagen: Levin and Munksgaard.

Porter, K. R. & Yegian, D. (1945). Some artifacts encountered in stained preparations of tubercle bacilli. II. Much granules and beads. *J. Bact.* **50**, 263.

Strangeways, T. S. P. (1924). *The Technique of Tissue Culture 'in vitro'*. Cambridge: Heffer and Sons Ltd.

Sweaney, H. (1928). The granules of the tubercle bacillus. *Amer. Rev. Tuberc.* **17**, 53.

Wyckoff, R. W. G. (1934). A micro-motion picture study of the growth of tubercle bacilli from cold-blooded animals. *Amer. Rev. Tuberc.* **24**, 389.

EXPLANATION OF PLATES

Cover-slip cultures of *Mycobacterium avium* grown on embryo-extract plasma clots and photographed with phase-contrast microscopy. Incubated at 37·5°.

Plate 1

Filamentous type of growth

Fig. 1. Starting preparation of a 5-week old culture (CG 1). Filamentous growth type. Small rod-shaped forms and isolated mycelial elements. (×4000.)

Fig. 2. After 48 hr. incubation (CG 2). Process of germination. (×2500.)

Fig. 3. After 7 days incubation (CG 3). Filaments resulting from elongation of elements of Fig. 2. Note branched form and the grouping of the filaments. (×2500.)

Mycelial type of growth

Fig. 4. Starting preparation of 7-week old culture (CG 5). Branched mycelial form, aggregates of small elements and minute spherical particles (granular forms). (×2500.)

Fig. 5. 24 hr. (CG 5). Note the presence of micro-rods. (×2500.)

Fig. 6. 5 days (CG 5). Complex structure characteristic of mycelial phase of growth. Results from proliferation of original mycelial elements. (×2500.)

Plate 2

Fig. 7. 8 days (CG 5). Mycelium disintegrating and releasing micro-rods. (×2500.)

Fig. 8. 14 days (CG 5). Micro-rods of fig. 7 after elongation. (×2500.)

Fig. 9*a*,*b*. 5 days (CG 5). First stage in spherical colony formation. Small nests of fine filaments. Note 'angular growth type'. (×2500.)

Fig. 10. 5 days (CG 5). Sprouting mycelium beginning to form small nest of filaments. Other parts of mycelium becoming swollen and dense. (×2500.)

Fig. 11. 13 days (CG 5). At a more advanced stage the microcolony assumes a spherical shape. Note rod-shaped inclusions within original mycelium. (×2500.)

Fig. 12. 13 days (CG 5). Fully formed spherical colonies. Note beading of filaments giving a granular appearance. The elements are now strongly acid-fast. (×2500.)

Plate 3

Fig. 13. 6 days (CG 5). Longitudinal splitting of dense, swollen filaments resulting in cord formation. (*a*) ×4000; (*b*) ×2500.

Fig. 14. 13 days (CG 5). Association of a cord formation and spherical colony. They have originated in different parts of the same original mycelium. (×4000.)

PLATE 4

Fig. 15. 18 days (CG 5). Typical bacillary forms resulting from mycelial development. Note beaded elements and small, bipolar rods. (×4000.)

Fig. 16. 19 days (CG 5). Typical bacillary forms. Disintegrated mycelium still visible in background. Note that some of the rods are dividing by simple fission. (×4000.)

Fig. 17. 6 days (CG 5). Zoogleal forms showing pseudo-mycelial structure. (×4000.)

Fig. 18. 8 days (CG 5). Zoogleal or L-form. Dense bodies in amorphous background. (×4000.)

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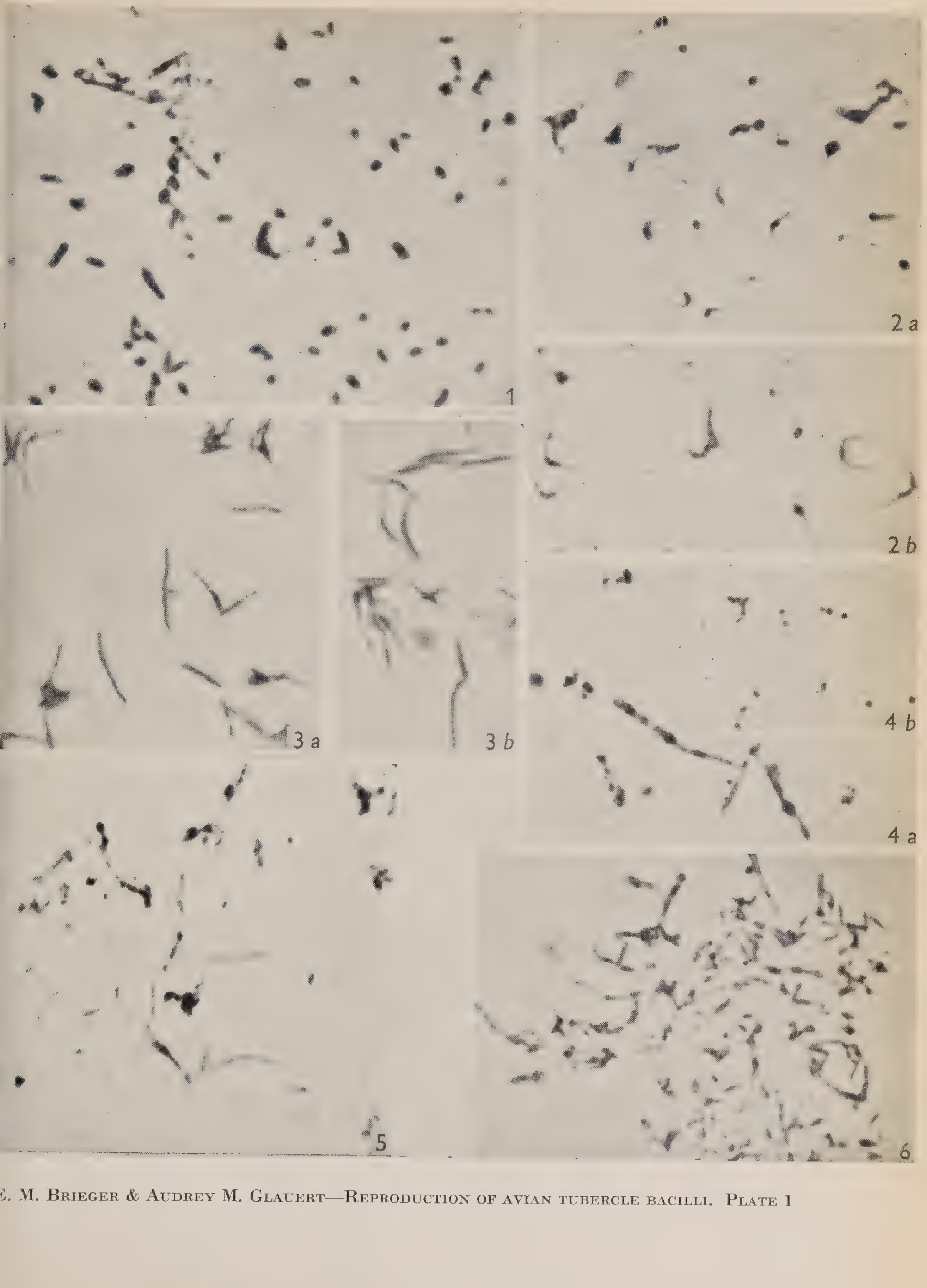
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E. M. Brieger & Audrey M. Glauert—Reproduction of avian tubercle bacilli. Plate 1

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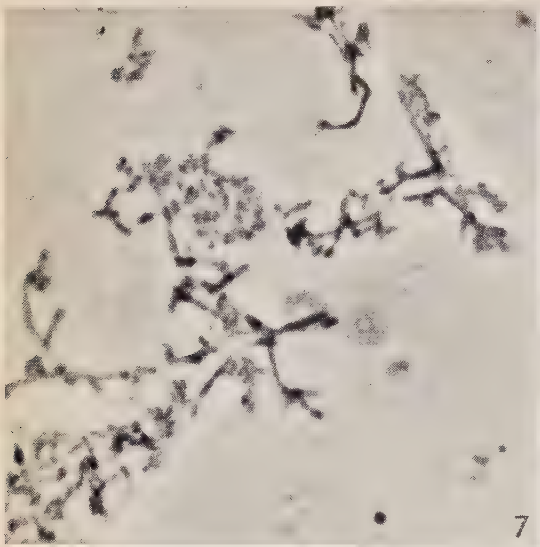
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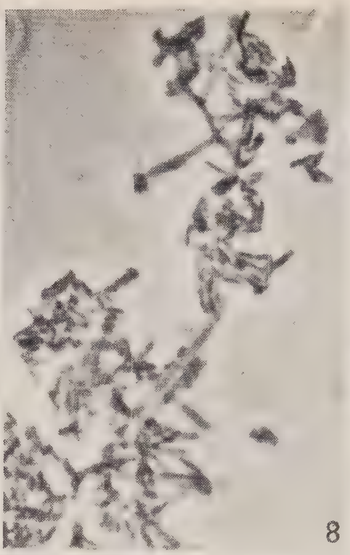
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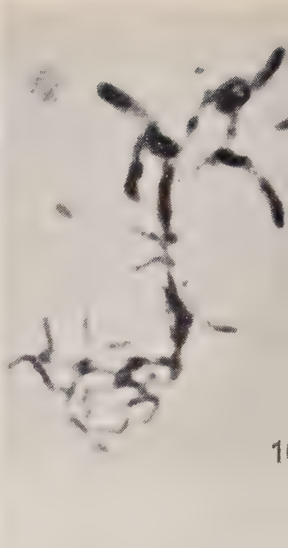
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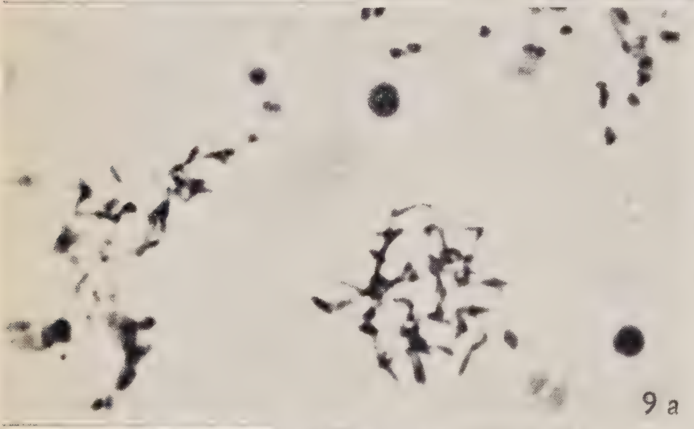
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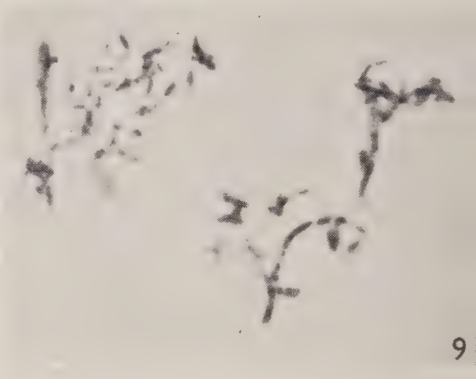
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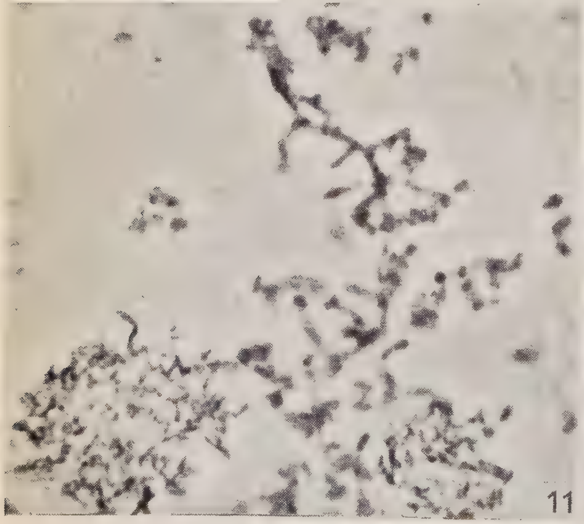
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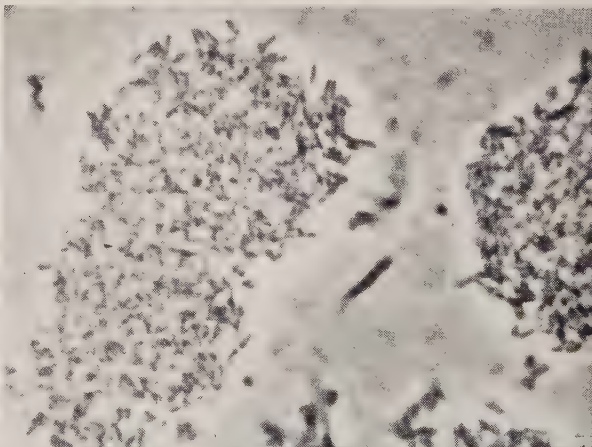
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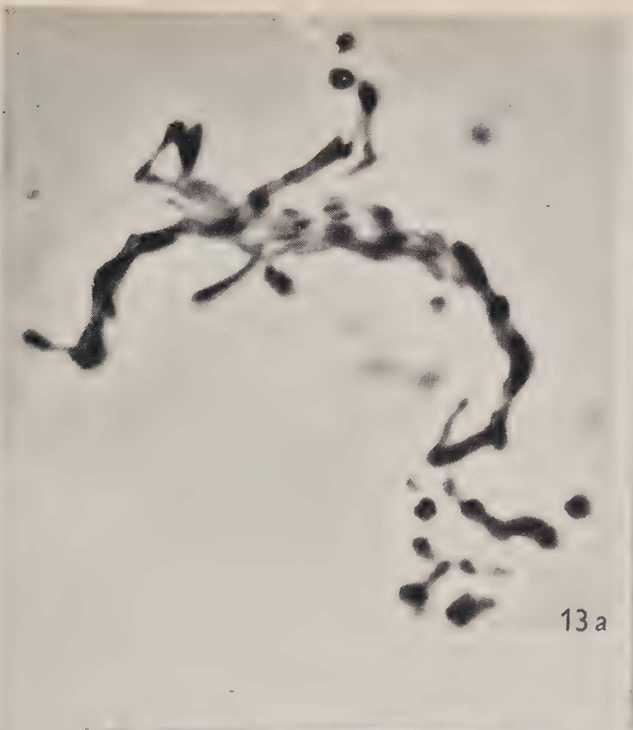
E. M. Brieger & Audrey M. Glauert—Reproduction of avian tubercle bacilli. Plate 2



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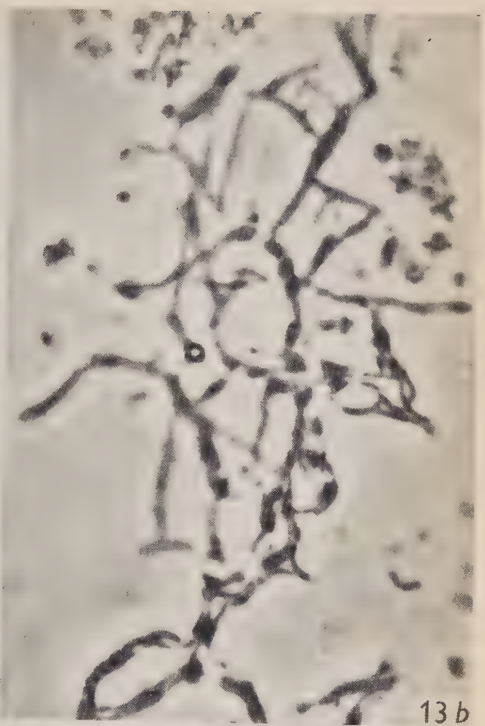

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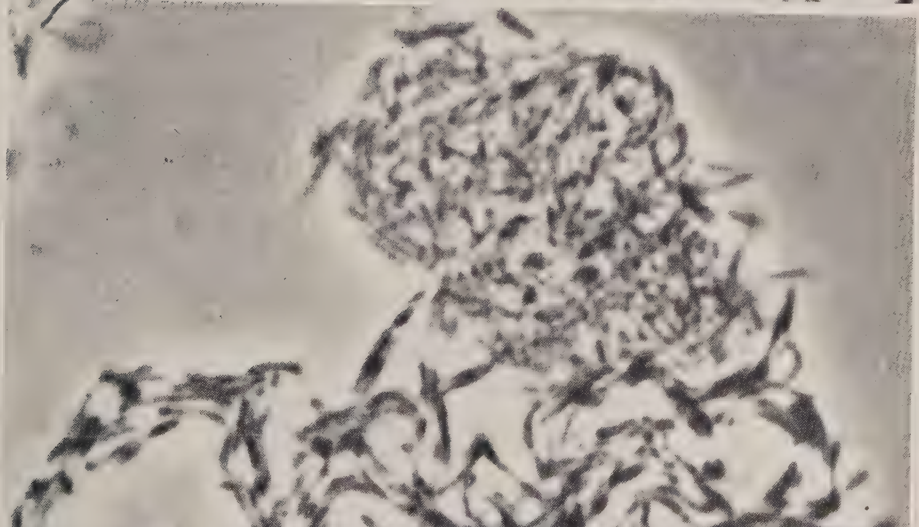

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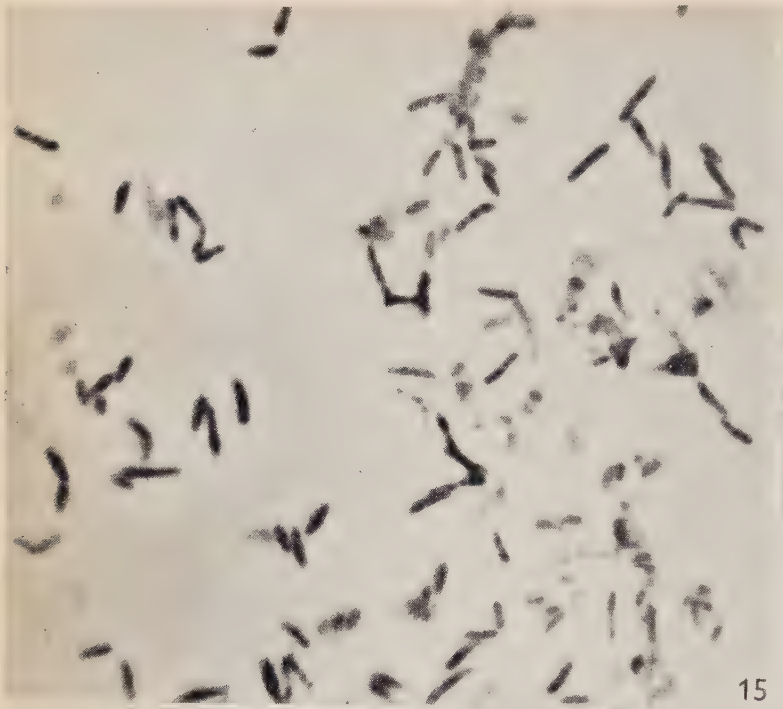
E. M. Brieger & Audrey M. Glauert—Reproduction of avian tubercle bacilli. Plate 3



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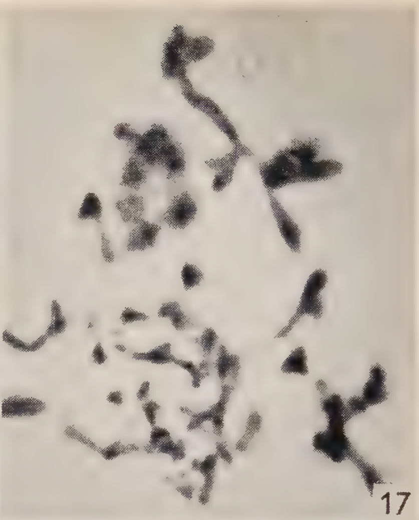

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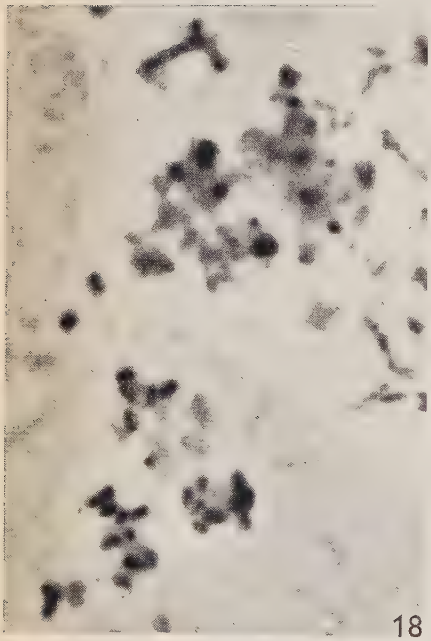

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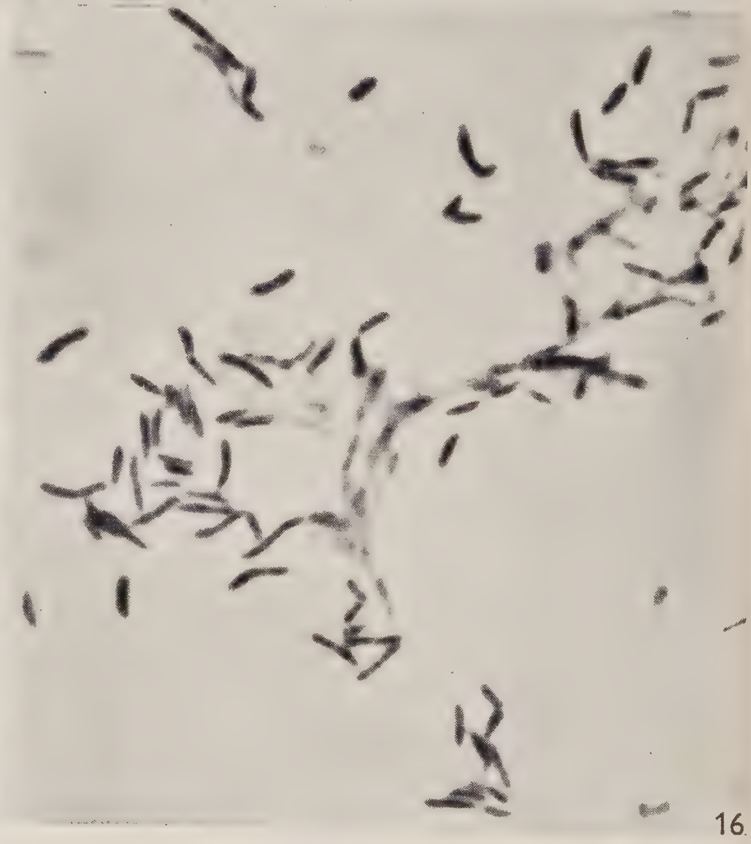

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E. M. Brieger & Audrey M. Glauert—Reproduction of avian tubercle bacilli. Plate 4

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The Stability of Antibiotics in Soils

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SUMMARY: Ten antibiotics have been included in this study; eight of them were metabolic products of fungi isolated from soils of the Bagshot Sand type. Their stability in Bagshot Sand soils and in a neutral garden loam has been investigated. Some were more stable than others, the rate of inactivation varied from soil to soil, but all exhibited a fair degree of stability in some of the soils. Four types of inactivation could be distinguished: (1) The natural pH of the soil was sometimes that at which the antibiotic was intrinsically unstable; this was noted specially with albidin, frequentin, gliotoxin, penicillin and viridin. (2) Inactivation caused by some form of biological activity, indicated by less rapid inactivation in heat-treated than in untreated soil, was observed with griseofulvin, mycophenolic acid and patulin. (3) Adsorption on the soil was noticeable only in the case of streptomycin, the only basic antibiotic studied; acid-washed sand was able to bind appreciable quantities of this antibiotic. (4) Some other form of inactivation, probably chemical in nature, was concerned in the inactivation of gladiolic acid, penicillin and streptomycin.

Discussion of the natural significance of antibiotics logically follows the discovery of their production by micro-organisms. Similarly, consideration of their possible role in soil ecology follows from the fact that many soil micro-organisms are capable of producing them. Prerequisites of a balanced judgement on the subject include information on: (*a*) the types of micro-organism commonly isolated from various types of soil; (*b*) the antibiotics produced by such soil micro-organisms; (*c*) the stability of such antibiotics in the soil from which their producers were isolated; (*d*) the mode of inactivation of these antibiotics where this is found to occur; (*e*) the possibilities of detecting antibiotic production and accumulation in natural soils; (*f*) competition between strains of micro-organisms which do and do not produce antibiotics; (*g*) the significance—if any—of antibiotics to the micro-organisms which produce them.

The present paper is concerned with the stability and mode of inactivation of antibiotics in soil. In order to relate this as closely as possible to conditions under which antibiotics might naturally be produced, the majority of the antibiotics chosen for study were produced by fungi isolated from the Bagshot Sand type of soil, in which production of antibiotics under natural conditions has been suspected (Brian, Hemming & McGowan, 1945; Rayner, 1945). Furthermore, among the soils selected for study were soils from different levels of a Bagshot Sand soil profile. Streptomycin has been included, although actinomycetes in general are scarce in these acid soils, to afford a basis of comparison with the somewhat similar studies of Siminoff & Gottlieb (1951) and Pramer & Starkey (1951).

MATERIALS

Soils. Four of the soils used were obtained from the profile of a pit dug in a typical Bagshot Sand podsol on Wareham Heath, Dorset. The fifth sample, included for comparison, was a neutral light garden soil from Welwyn, Hertfordshire. The origin and properties of these soils are shown in Table 1.

Table 1. *Description of soils used*

Sample	Horizon	Depth (in.)	Description	pH value Fresh	pH value Sterile
			Soils from profile from Wareham Heath, Dorset		
*	A_0	0–2	Litter and peat	—	—
Wareham I	A_1	2–6	Black sandy peat	4·2	4·1
*	A_2	6–10	Slightly leached grey sand	—	—
Wareham II	A_3	10–18	Leached sand	5·0	5·4
Wareham III	B_{1-2}	18–22	Podsol pan	4·2	4·3
Wareham IV	C	22–26	Brown sandy gravel—some clay	4·9	5·0
			Soil from Laboratory Garden, Welwyn, Hertfordshire		
Garden	—	0–3	Light neutral loam	7·2	7·2

* Soils from these horizons were not used in the experiment.

After collection the soils were air-dried, coarsely crushed and stored in tins at room temperature. The sand used in some experiments was washed for 24 hr. in running water, then for 12 hr. in *c.* N-HCl, and finally for another 36 hr. in running water. After this treatment very little clay remained and it had virtually no buffering capacity. Assays made as later described on aqueous extracts of these soils, at the concentration used in the experiments, showed no antifungal or antibacterial activity. Thus, all activity measured in the experiment can only have been caused by the antibiotic added.

Antibiotics. The following antibiotics were studied: albidin (Curtis & Grove, 1947), frequentin (Curtis, Hemming & Smith, 1951), gladiolic acid (Brian, Curtis & Hemming, 1948), gliotoxin (Brian, 1946), griseofulvin (Brian, Curtis & Hemming, 1946), mycophenolic acid, patulin, viridin (Brian & McGowan, 1945), penicillin and streptomycin. All these, except the last two, were prepared in these laboratories from fungi isolated from soils of the Bagshot sand type. These organisms are listed in Table 2.

Frequentin, patulin, penicillin and streptomycin are soluble in cold water, so that solutions of the required concentration could easily be prepared. The remaining antibiotics being only sparingly soluble in water are most conveniently brought into solution in a small quantity of a water-miscible organic solvent followed by dilution with water. To complicate the interpretation of results by the introduction of organic solvents into the system was undesirable, as these might well alter the microbiological activity of soil, so this method was not used. Gladiolic acid and mycophenolic acid were obtained as aqueous solutions of their sodium salts by solution in sodium hydroxide of appropriate strength. Gliotoxin and viridin were unstable in simple aqueous solution and were prepared in a dilute McIlvaine buffer by refluxing in a citric acid solution of appropriate strength, and adding the phosphate of the buffer in appropriate quantities later. Albidin was dissolved in the cold in water slightly acidified with HCl. Griseofulvin was dissolved by refluxing in water. The concentration of griseofulvin, viridin and albidin solutions was determined, after preparation of the solutions, by biological assay.

Table 2. *Origin, potency and method of assay of antibiotics*

Antibiotic	Produced from	Potency	Assay
Albidin	*Penicillium albidum* Sopp (BRL 248)	0·6	BA
Frequentin	*P. frequentans* Westl. (BRL 737)	3·0	BA
Gladiolic acid	*P. gladioli* McCull & Thom (BRL 59)	7·0	BA
Gliotoxin	*P. terlikowskii* Zal. (BRL 536) *Trichoderma viride* Pers. ex Fr. (BRL 211)	3·5	BA
Griseofulvin	*P. nigricans* (Bainier) Thom (BRL 250)	1·1	BA
Mycophenolic acid	*P. stoloniferum* Thom (BRL 438)	0·4	BA
Patulin	*P. expansum* Link (BRL 440)	—	SCP
Viridin	*Trichoderma viride* Pers. ex Fr. (BRL 213)	0·06	BA
Penicillin	*P. chrysogenum* Thom	1690 units/mg.	SCP
Streptomycin	*Streptomyces griseus* (Krainsky) Waksman & Henrici	423 units/mg.	SCP

Notes. The numbers appearing in parentheses after names of organisms are the accession numbers in the culture collection of Butterwick Research Laboratories (BRL). Penicillin G was supplied by I.C. Pharmaceuticals Ltd. Streptomycin was supplied by Boots Pure Drug Co. Ltd. Potencies refer (except in the case of streptomycin and penicillin) to least concentrations (μg./ml.) preventing germination of *Botrytis allii* spores or (in case of griseofulvin) producing curling of *Botrytis allii* hyphae. Methods of assay: BA = *Botrytis allii* spore germination test; SCP = *Bacillus subtilis* cylinder plate assay.

METHODS

Experimental layout. The stability of each antibiotic in a McIlvaine buffer (0·01 M-citric acid + 0·02 M-Na_2HPO_4) over a pH range of 3–7 was first determined. Next, the stability of similar solutions was determined in the presence of acid-washed sand. Finally, the stability of solutions of the antibiotic was determined in the presence of each of the five soils previously described, using both air-dried but otherwise untreated soil (subsequently referred to as fresh) and soil autoclaved at 15 lb./sq. in. for 25 min. (subsequently referred to as partially sterile). In most cases both water solutions and buffer solutions of the antibiotic were exposed to fresh soil, the buffer being of a pH value close to the natural pH value of the soil; buffer solutions only were exposed to partially sterile sand and soil. For reasons already explained experiments with viridin and gliotoxin could only be carried out in buffer. Since the buffers used were very dilute, the pH value occasionally drifted slightly during the course of the experiment.

In the soil and sand experiments 250 ml. antibiotic solution was placed in a 'Glaxo' culture vessel and 25 g. (air-dry weight) of soil added. This gave a 2 mm. layer of soil or sand with a 10 mm. supernatant layer of solution. It is appreciated that such conditions might seem to be unduly anaerobic and that an excessive proportion of antibiotic solution was exposed to a given volume of soil. It was thought, however, that under such conditions processes of inactivation would be slowed down and more readily studied. Moreover, preliminary experiments in which this condition was compared with the more obviously aerobic conditions of a soil percolator (Jefferys & Smith, 1951), indicated that there was little difference between the two experimental conditions. The static technique, being far simpler to handle, was therefore adopted.

In all experiments two concentrations of antibiotic were used. The lower was the lowest that could be conveniently assayed and the higher was in all cases ten times greater than the lower.

Methods of assay. Samples for assay were aseptically taken and every effort was made to remove at the same time a part of the soil present, in order to maintain an approximately constant ratio between soil and solution. Assays were made within 1 hr. of setting up the experiment and thereafter at suitable intervals. All samples were filtered through No. 5 Whatman filter-paper before assay.

Patulin, penicillin and streptomycin were assayed by the cylinder plate technique (Foster & Woodruff, 1944). The test organism in each case was *Bacillus subtilis*, a strain received from I.C. Pharmaceuticals Ltd. being used for assay of patulin and penicillin, and a strain (NCTC 7241) obtained from Boot's Pure Drug Co. Ltd. for streptomycin. The assay agar contained peptone, Lab-Lemco meat extract and Marmite. Standard curves were constructed daily and the concentrations of the antibiotics in the unknown solutions estimated graphically from these curves.

The remaining antibiotics were assayed by a serial dilution spore germination test using conidia of *Botrytis allii*. Brian & Hemming (1945), describing the method, used the inhibition of germination as the end-point of the assay. In the present work, the end-point used was based on loss of the germ tubes, which occurs at higher dilutions than inhibition of spore germination. This results in a more sensitive assay. Titres are expressed in 'dilution units'. This term is the number of consecutive twofold dilutions, through which inhibition of germination or stunting of germ-tubes can be recognized. These units are proportional to the logarithm of the concentration of the active material.

The assay of griseofulvin is different in that the limiting concentrations needed to produce the various characteristic morphological changes in germ-tubes were recorded; results are presented similarly in dilution units.

RESULTS

These are expressed in Figs. 1–19, and commented on in the text below.

Albidin

Albidin is unstable throughout the range pH 3·3–6·9 in buffer solution (Fig. 1). In the more acid solution (pH 3·3–4·8), after an initial rapid fall in activity, a steady level of activity is reached. This can be interpreted as meaning that albidin breaks down to a second antifungally active material of greater stability at low pH. At pH 5·9 and above, the active degradation product is also unstable. Sand had little effect on the rate of inactivation of albidin. The soils varied in their effect (Fig. 2). Wareham II and IV had little effect. In Wareham I, and less noticeably in Wareham III, the rate of inactivation in fresh soil is greater than would be expected merely from the pH of the solution, though partially sterile soil had little effect. This can best be interpreted as due to microbial activity; such differential inactivation in fresh and partially sterile soil is termed 'biological inactivation' in the following pages. In the garden soil inactivation is extremely rapid, as would be expected from the high pH; indications of biological inactivation are slight.

Frequentin

Buffer solutions of frequentin (Fig. 3) are highly stable in the range pH 3·3–4·6. Activity is slowly lost at pH 5·7 and more rapidly at pH 6·9. Sand has no effect on the rate of inactivation at neutrality, but at lower pH it may somewhat increase the rate of inactivation (Fig. 3). The rate of inactivation in Wareham II and IV soils (Fig. 4) is similar to that in buffer or in buffer and sand. In Wareham I and III soils inactivation is more rapid, and in Wareham I the greater rate of inactivation in fresh soil as compared with the rate in partially sterile soil, suggests that here again biological inactivation is taking place. Inactivation is very rapid in garden soil; this is mainly attributable to the high pH, but there is some indication of biological inactivation in the fresh soil.

Gladiolic acid

Gladiolic acid solutions in buffer (Fig. 5) are fairly stable in the range pH 3·4–7·0 and addition of sand has no effect. Once again Wareham II and IV soils had no effect. Inactivation at a much greater rate takes place in Wareham I and III and garden soils (Fig. 6); the rate of inactivation is little greater in fresh than in partially sterile soil. This indicates that in these soils inactivation is mainly due to some cause other than microbial decomposition or inactivation attributable to pH.

Gliotoxin

Buffer solutions of gliotoxin (Fig. 7) are highly stable at pH 3·4 and 4·9. Above pH 4·9 slow inactivation takes place, being more rapid the higher the pH. Sand had little if any effect on the rate of inactivation. In the presence of all the soils such inactivation as was observed (Fig. 8) could be explained by the pH of the system.

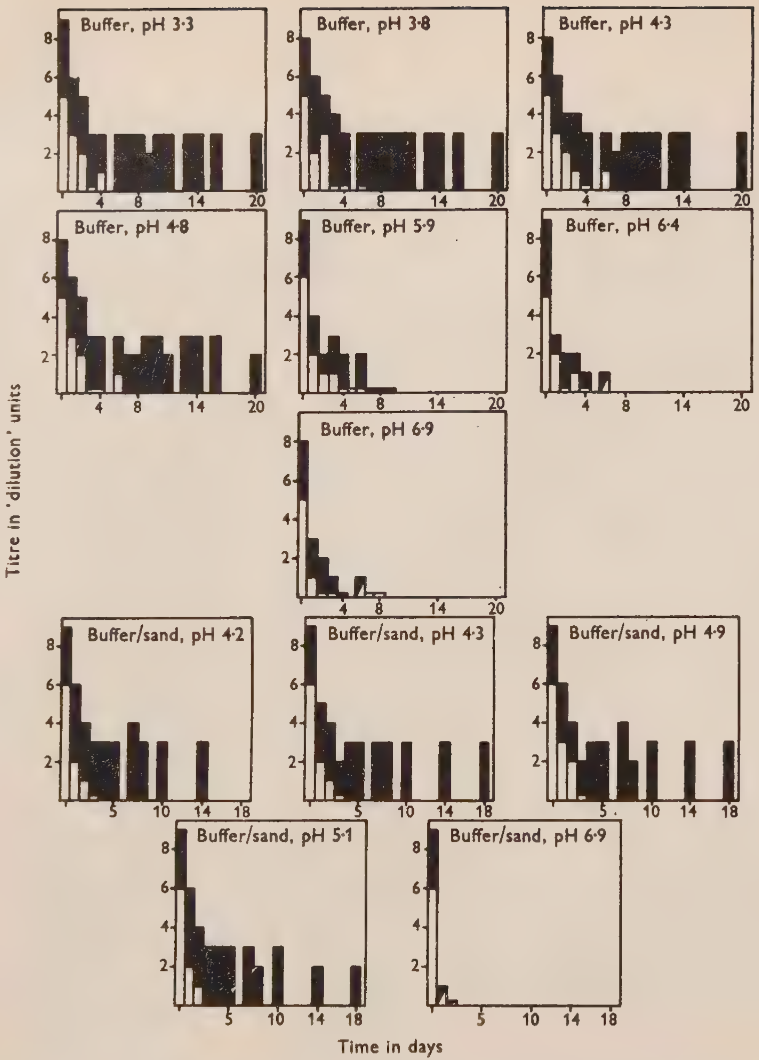


Fig. 1. Fungistatic activity of solutions of albidin (initially *c.* 30 μg./ml., ■, and *c.* 3 μg./ml., □) stored at 25° in buffer in the presence and absence of sand.

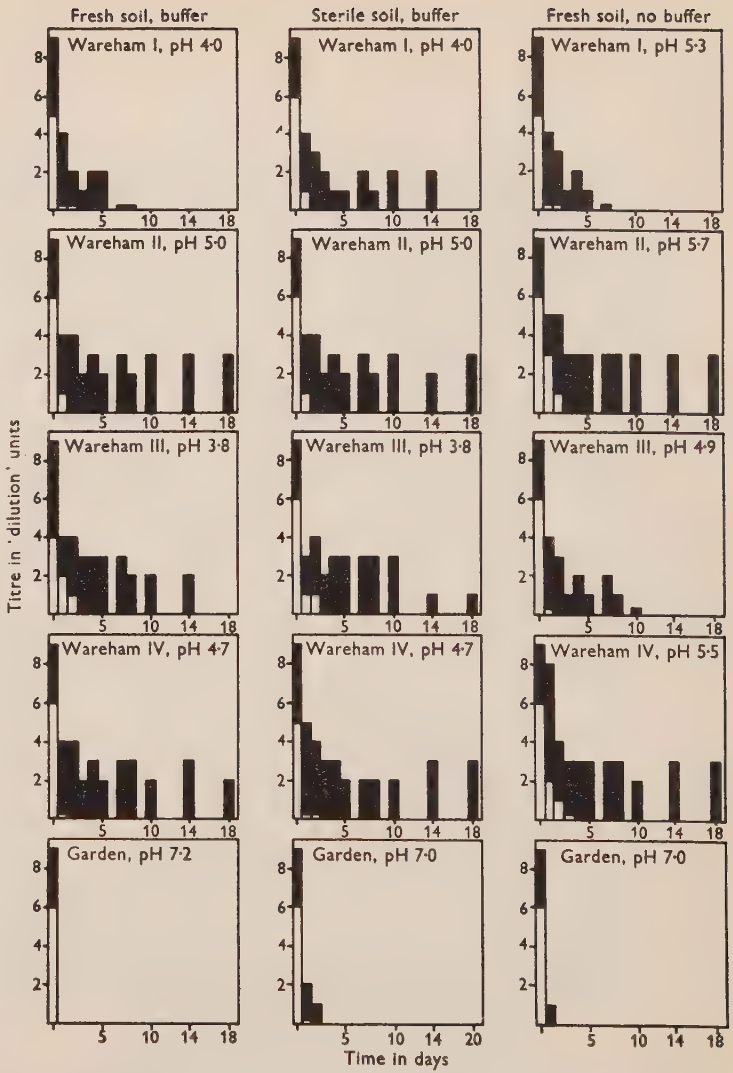


Fig. 2. Fungistatic activity of solutions of albidin (initially *c.* 30 μg./ml., ■, and 3 μg./ml., □) stored at 25° in contact with variously treated soils.

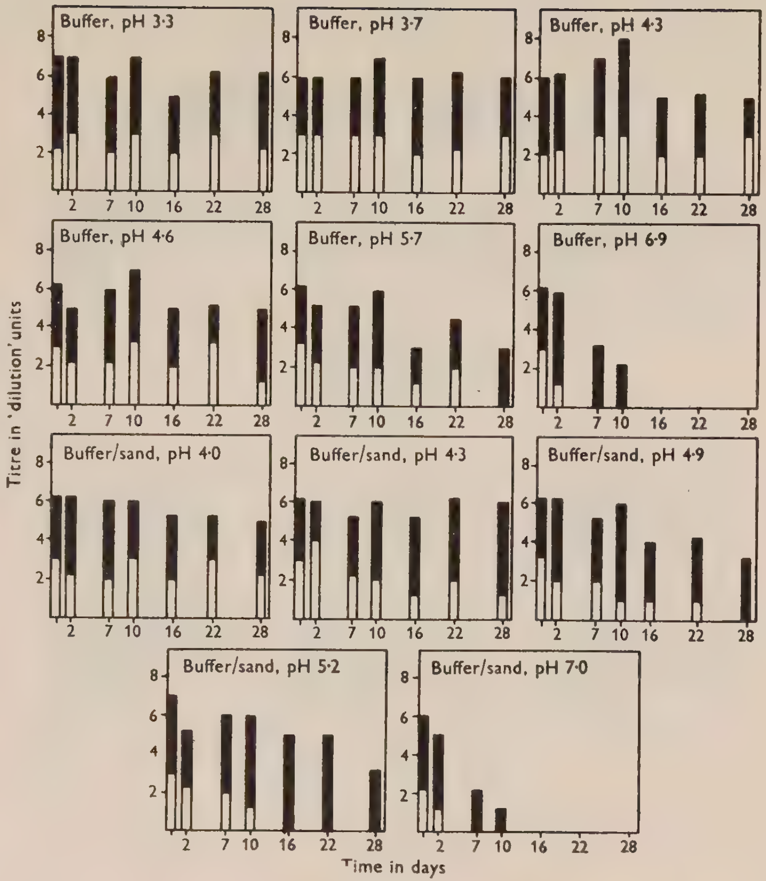


Fig. 3. Fungistatic activity of solutions of frequentin (initially *c.* 100 μg./ml., ■, and *c.* 10 μg./ml., ☐) stored at 25° in buffer in the presence and absence of sand.

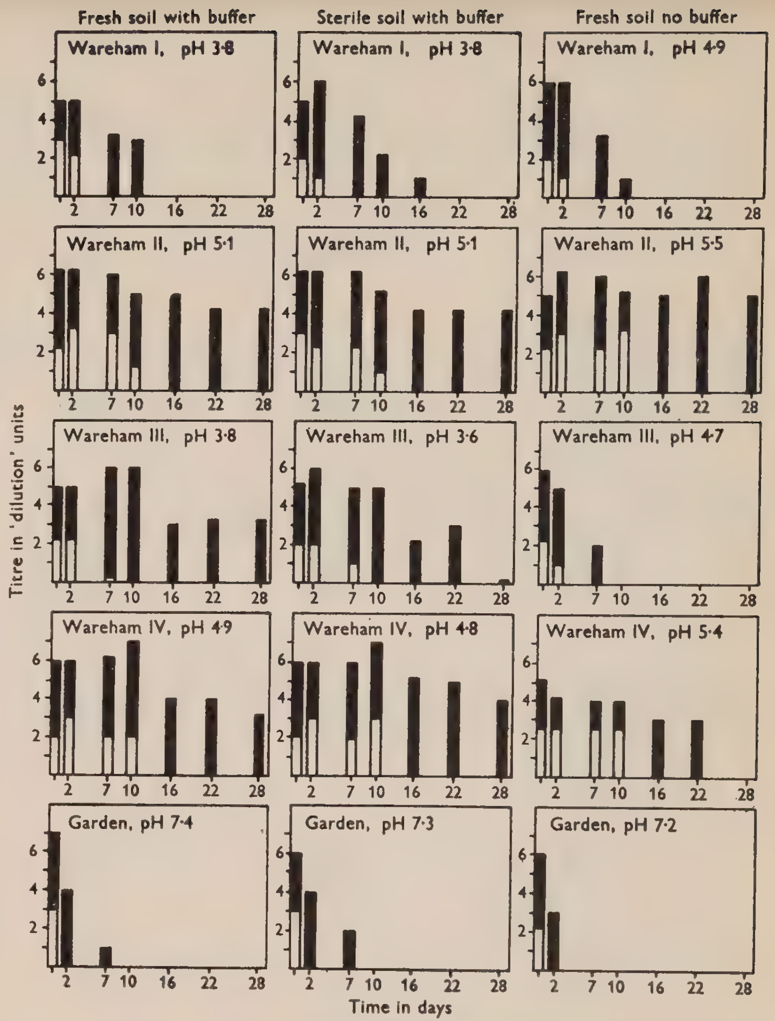


Fig. 4. Fungistatic activity of solutions of frequentin (initially *c.* 100 μg./ml., ■, and *c.* 10 μg./ml., ☐) stored at 25° in contact with variously treated soils.

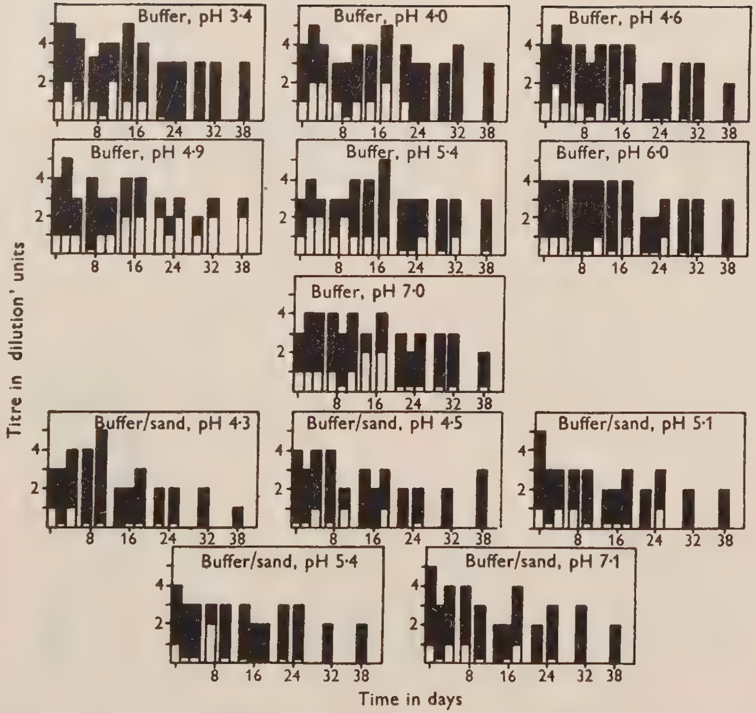


Fig. 5. Fungistatic activity of solutions of gladiolic acid (initially *c.* 30 μg./ml., ■, and *c.* 3 μg./ml., ☐) stored at 25° in buffer in the presence and absence of sand.

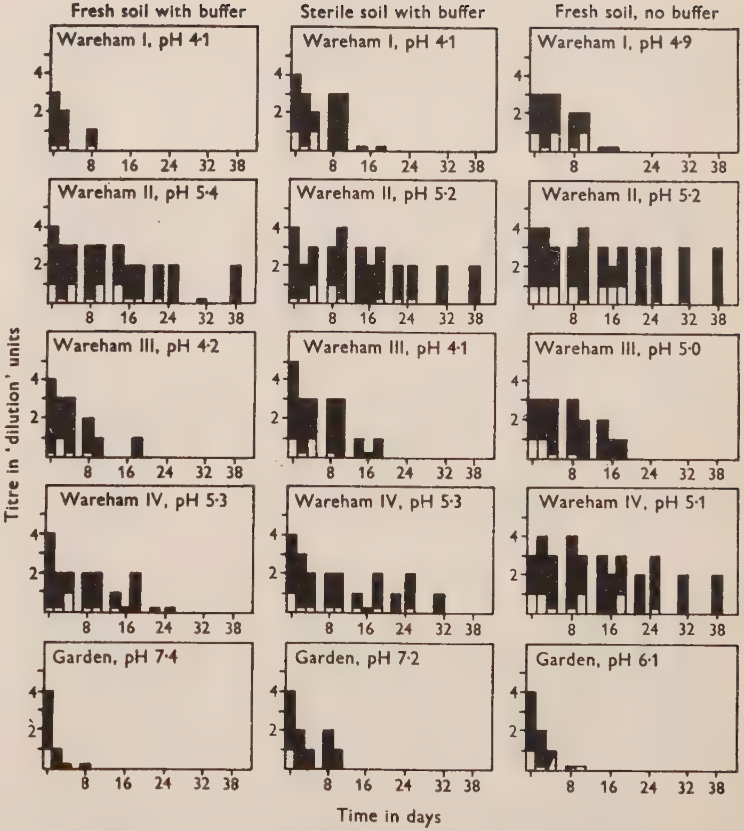


Fig. 6. Fungistatic activity of solutions of gladiolic acid (initially *c.* 30 μg./ml., ■, and *c.* 3 μg./ml., ☐) stored at 25° in contact with variously treated soils.

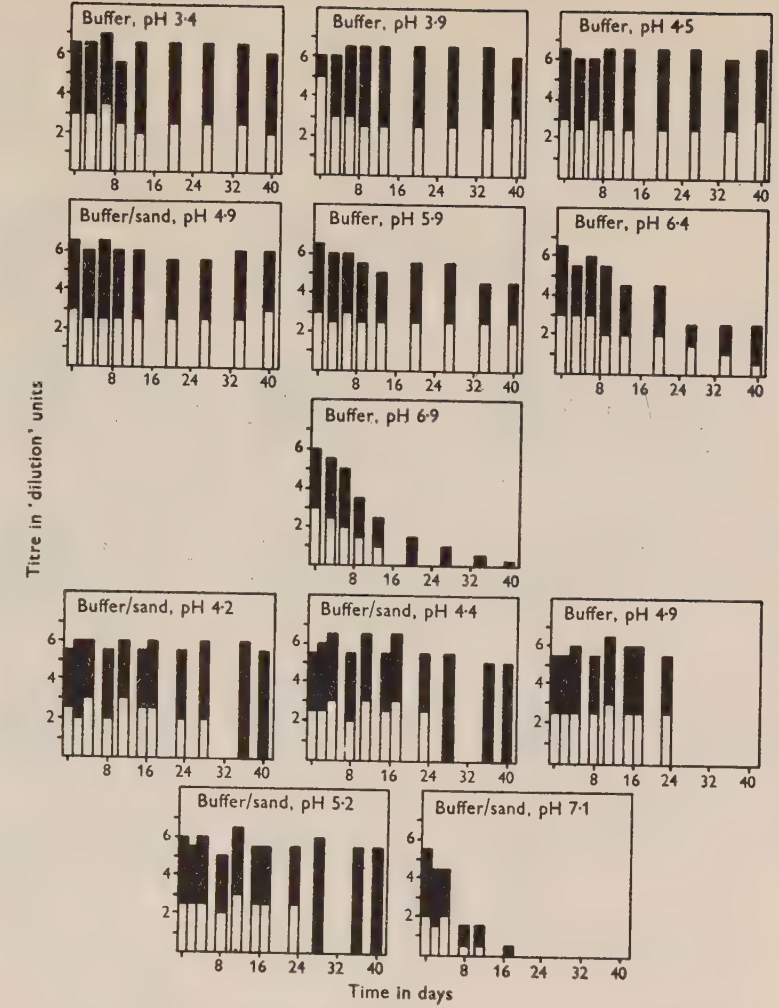


Fig. 7. Fungistatic activity of solutions of gliotoxin (initially *c.* 200 μg./ml., ■, and *c.* 20 μg./ml., ☐) stored at 25° in buffer in the presence and absence of sand.

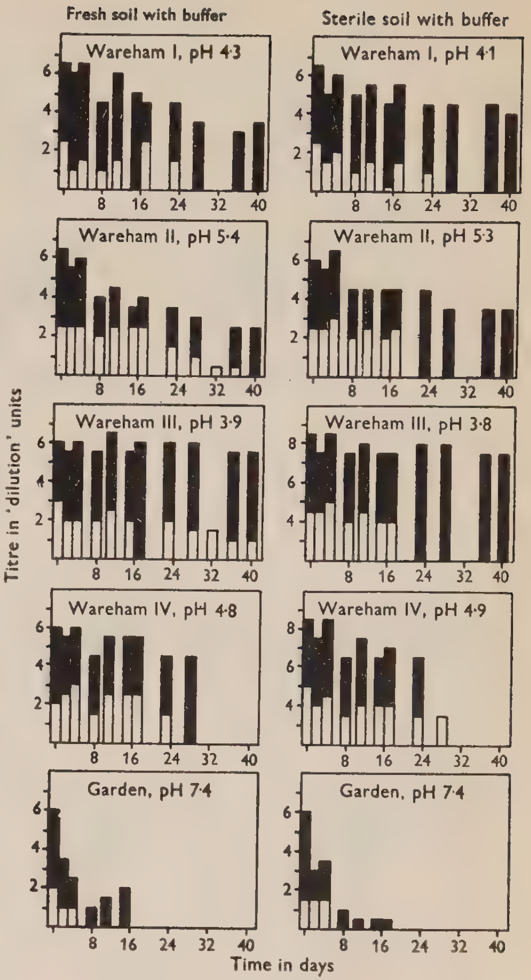


Fig. 8. Fungistatic activity of solutions of gliotoxin (initially *c.* 200 μg./ml., ■, and *c.* 20 μg./ml., ☐) stored at 25° in contact with variously treated soils.

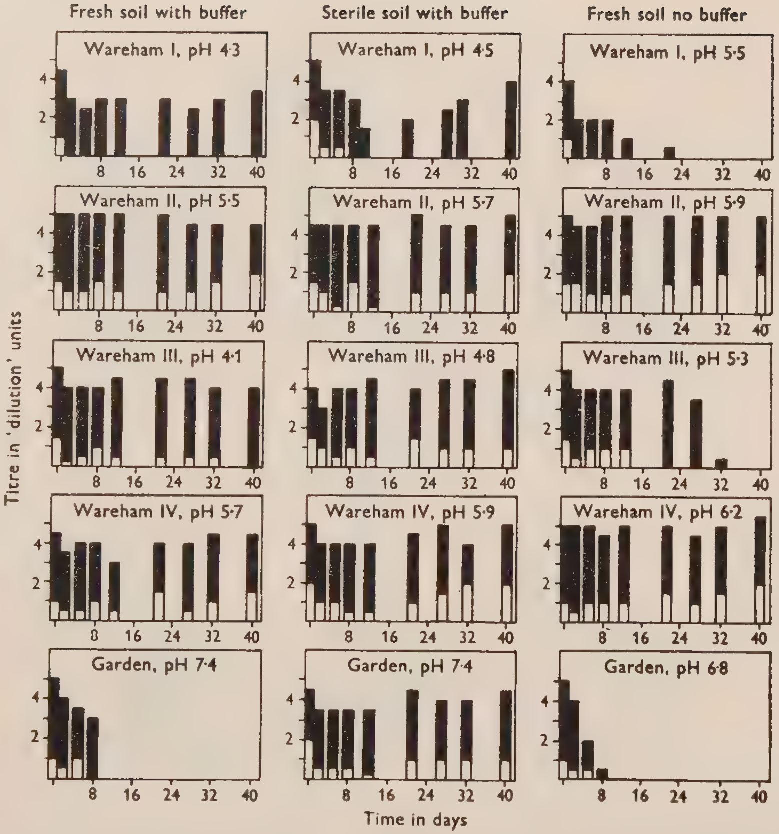


Fig. 9. Fungistatic activity of solutions of griseofulvin (initially *c.* 30 μg./ml., ■, and *c.* 3 μg./ml., □) stored at 25° in contact with variously treated soils.

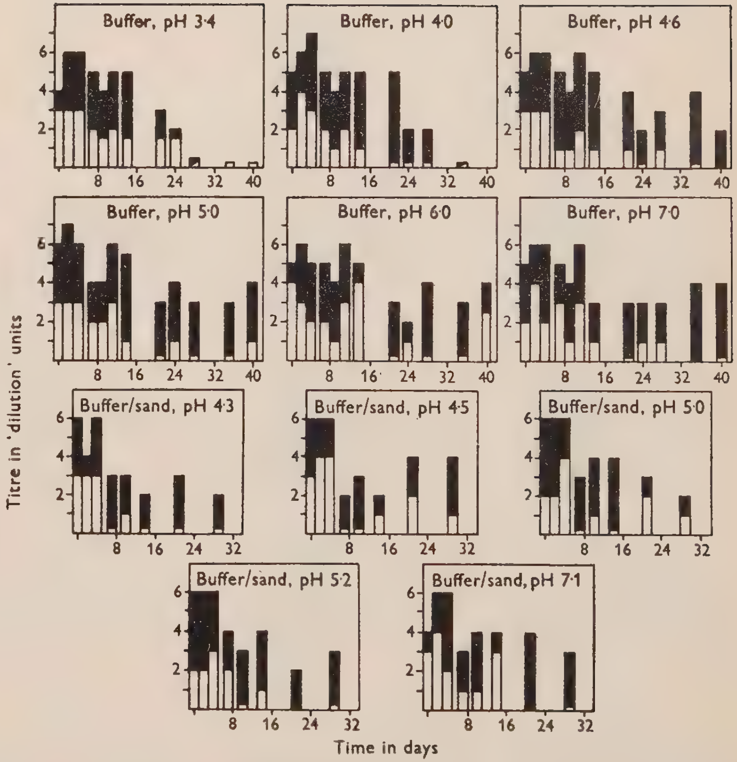


Fig. 10. Fungistatic activity of solutions of mycophenolic acid (initially *c.* 20 μg./ml., ■, and *c.* 2 μg./ml., □) stored at 25° in buffer in the presence and absence of sand.

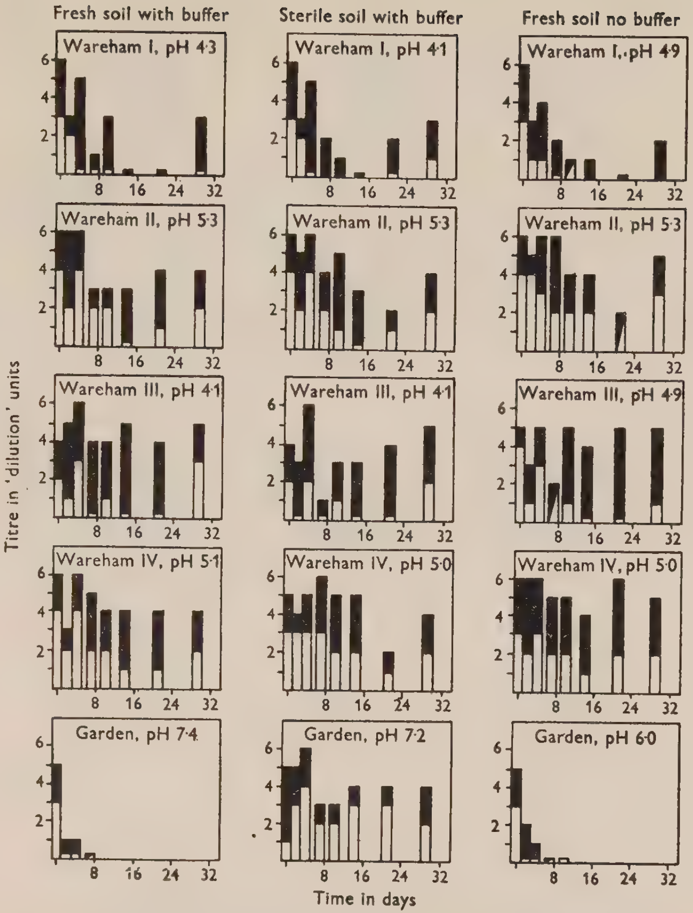


Fig. 11. Fungistatic activity of solutions of mycophenolic acid (initially *c.* 20 μg./ml., ■, and *c.* 2 μg./ml., □) stored at 25° in contact with variously treated soils.

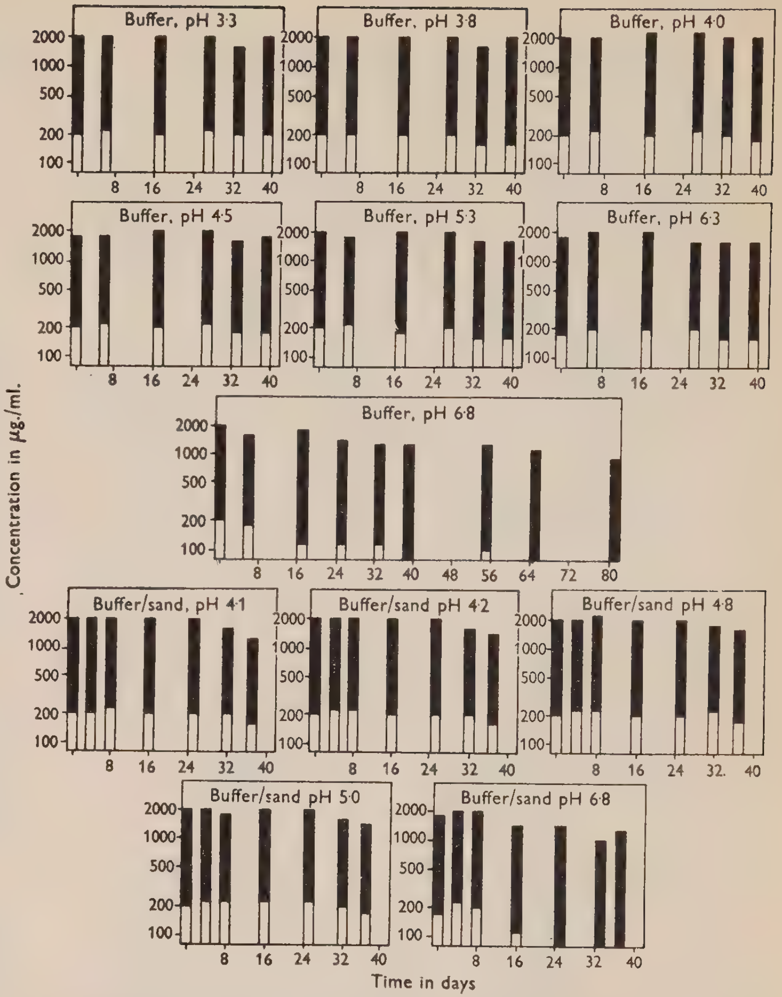


Fig. 12. Estimated concentrations of solutions of patulin (initially *c.* 2000 μg./ml., ■, and *c.* 200 μg./ml., □) stored at 25° in buffer in the presence and absence of sand.

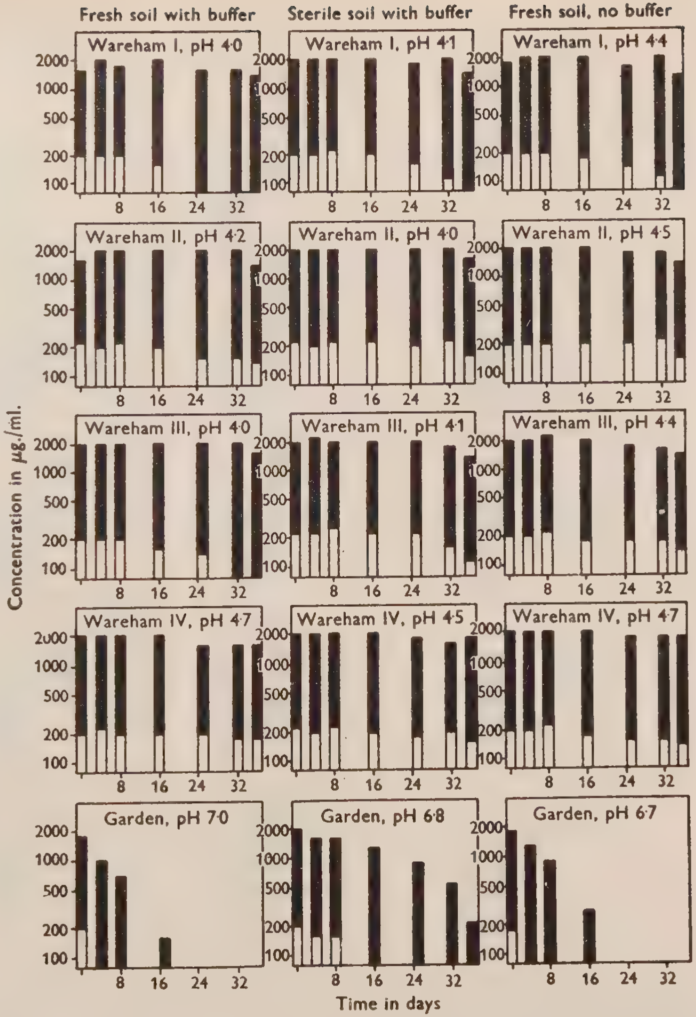


Fig. 13. Estimated concentrations of solutions of patulin (initially *c.* 2000 μg./ml., ■, and *c.* 200 μg./ml., ☐) stored at 25° in contact with variously treated soils.

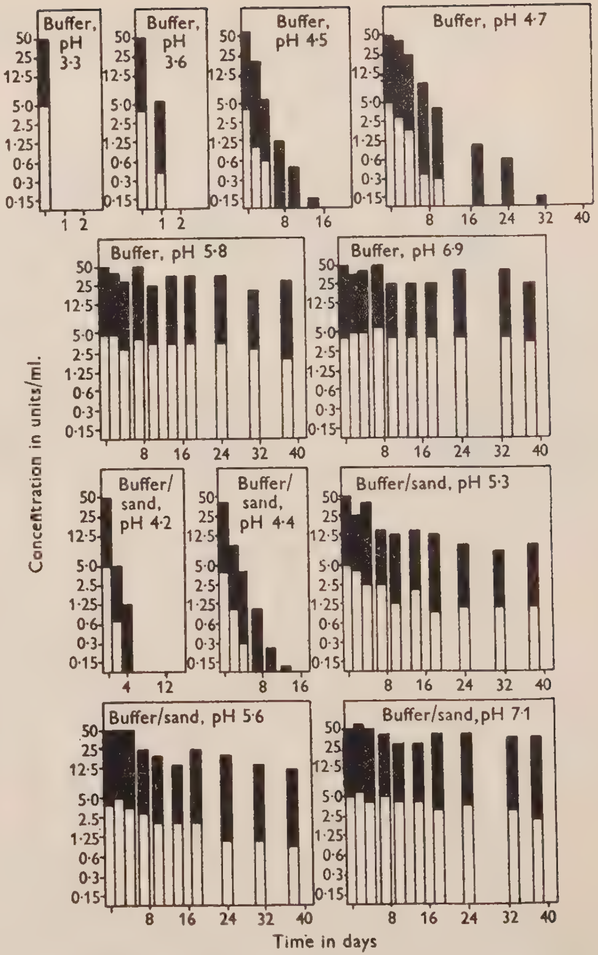


Fig. 14. Estimated concentrations of solutions of penicillin (initially *c.* 50 units/ml., ■, and *c.* 5 units/ml., ☐) stored at 25° in buffer in the presence and absence of sand.

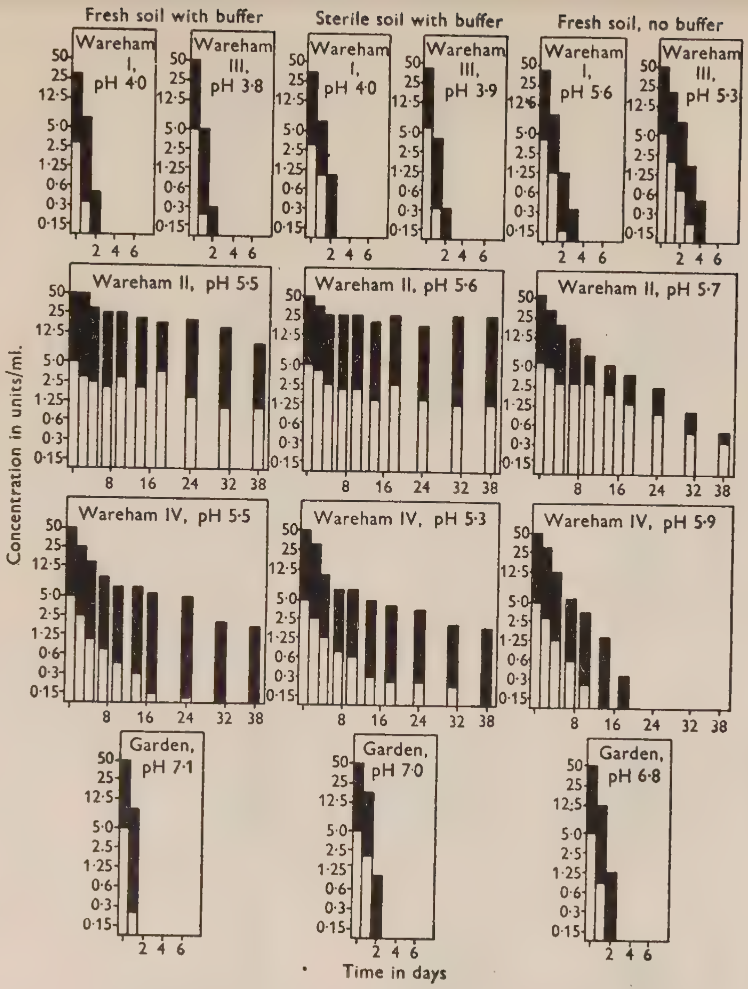


Fig. 15. Estimated concentrations of solutions of penicillin (initially *c.* 50 units/ml., ■, and *c.* 5 units/ml., ☐) stored at 25° in contact with variously treated soils.

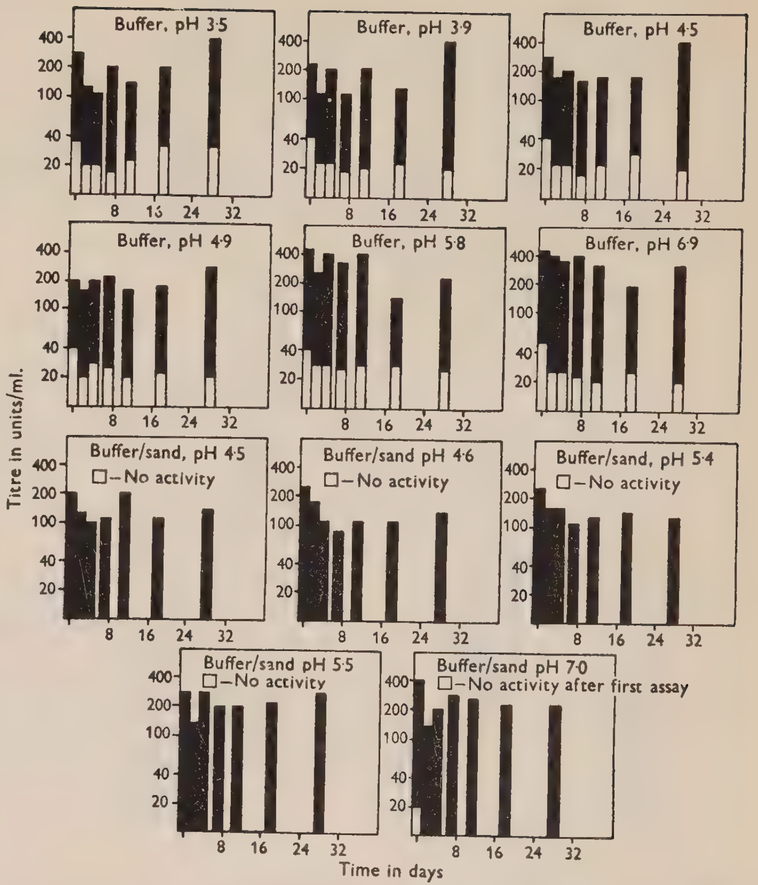
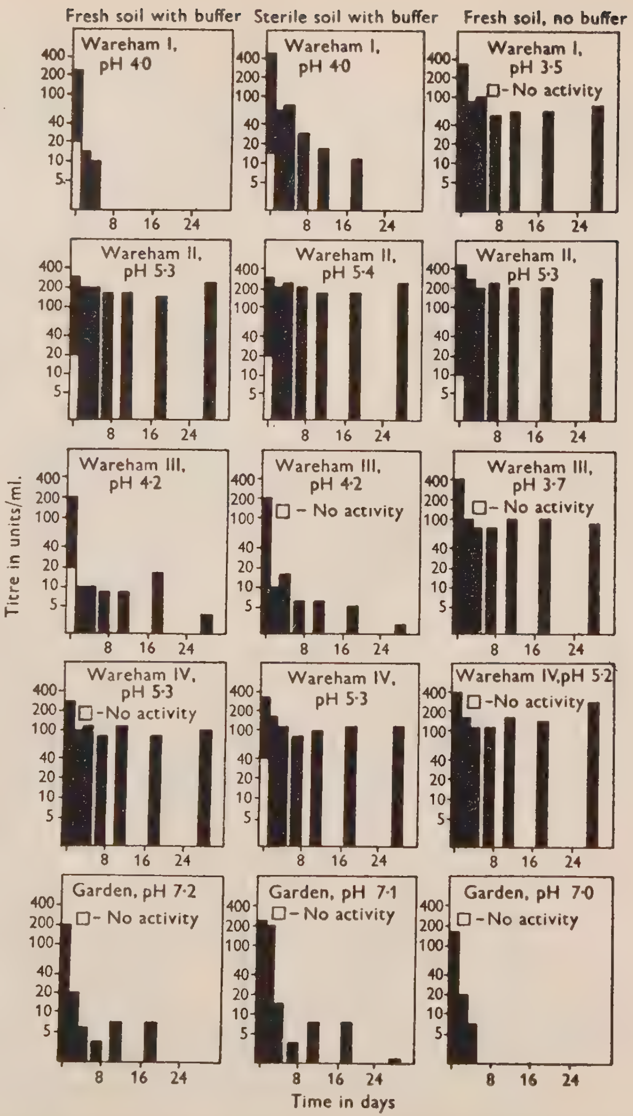


Fig. 16. Estimated concentrations of solutions of streptomycin (initially *c.* 400 units/ml. and *c.* 40 units/ml., ☐) stored at 25° in buffer in the presence and absence of sand.



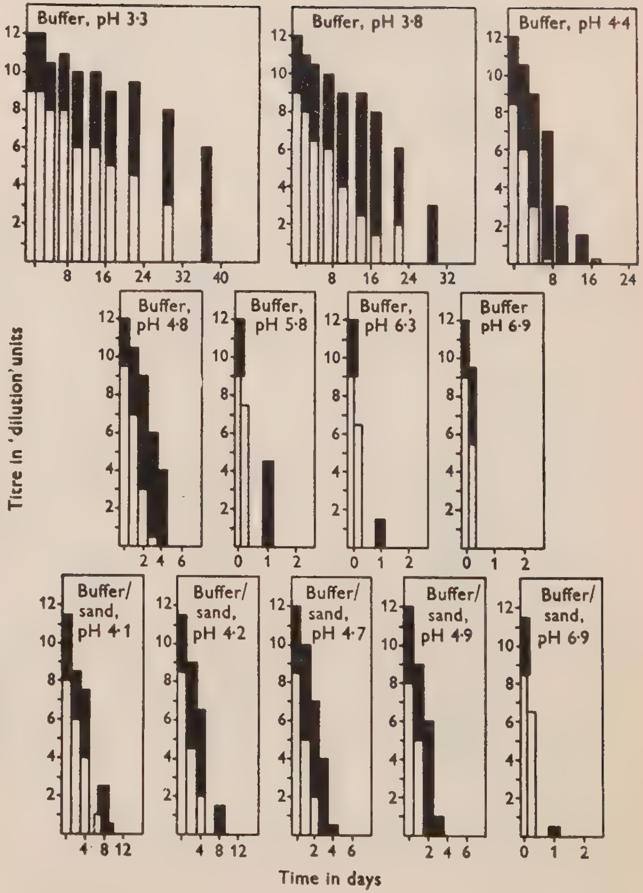


Fig. 17. Estimated concentrations of solutions of streptomycin (initially c. 400 units/ml., ■,

Fig. 18. Fungistatic activity of solutions of viridin (initially c. 100 μg./ml., ■, and c. 10 μg./ml., □) stored at 25° in

Griseofulvin

Solutions of griseofulvin were completely stable over the range pH 3·4–7·0 and addition of sand had no effect, and accordingly detailed data are not presented. Rapid biological inactivation occurred in the fresh garden soil (Fig. 9). It was stable in Wareham II and IV soils; it was also stable in Wareham III, though in the solutions in water an inexplicable rapid loss in activity took place towards the end of the experiment. In Wareham I soil the more concentrated solutions in buffer showed no indication of biological inactivation, and quite high titres were recorded after 40 days; however, the rather lower general level of activity as compared with that recorded in buffer solution with no soil present, contrasted with the rapid and complete disappearance of griseofulvin from the less concentrated solutions, suggests that some such process as a physical adsorption took place.

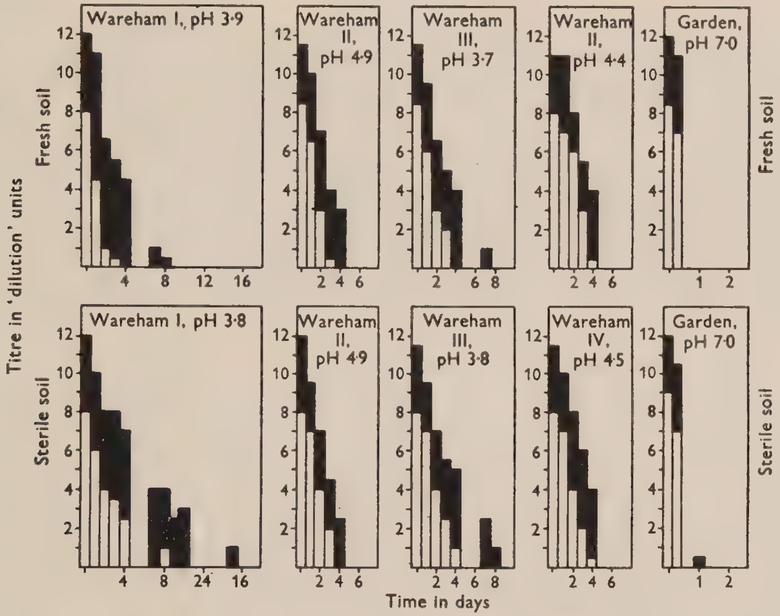


Fig. 19. Fungistatic activity of solutions of viridin (initially *c.* 100 μg./ml., ■, and *c.* 10 μg./ml., □) stored at 25° in contact with variously treated soils.

Mycophenolic acid

Mycophenolic acid solutions in buffer at pH 4·6–7·0 are only very slowly inactivated; at pH 4·0 and below inactivation is rather more rapid (Fig. 10). Sand had no effect. The antibiotic was very stable in Wareham II, III and IV soils (Fig. 11). In the Wareham I soil, whether fresh or partially sterile, activity gradually disappeared over 16 days and thereafter began to reappear. In fresh garden soil rapid biological inactivation took place.

Patulin

Patulin was almost completely stable in buffer solution in the pH range 3·3–6·3; at pH 6·8 slow inactivation took place (Fig. 12). Sand had no appreciable effect on the rate of inactivation. It was very stable in all soils of the Wareham series (Fig. 13). Inactivation was more rapid in partially sterile garden soil and was greatly accelerated in fresh garden soil, indicating a considerable biological element among the factors causing inactivation.

Penicillin

Penicillin is stable at neutrality but decreases in stability as the pH is lowered (Fig. 14). This accords with previously published data (Florey, Chain, Heatley, Jennings, Sanders, Abraham & Florey, 1949), though it was rather more stable at pH 5·8 and 6·9 in our experiments than would have been expected from the earlier data. Sand had no effect on the rate of inactivation. The garden soil, whether fresh or partially sterile, rapidly inactivated penicillin, in spite of a pH favourable to stability. Inactivation was rapid in Wareham I and III, as would have been expected from the low pH. In Wareham II and IV it was surprisingly stable with but the slightest indication of biological inactivation (Fig. 15).

Streptomycin

Over the pH range 3·5–6·9, no activity was lost from buffer solutions of streptomycin over the period of the experiment (Fig. 16). This agrees with results quoted by Waksman (1949). Addition of sand to the less concentrated streptomycin solutions (40 units/ml.) caused an immediate and complete loss of activity. At the lower pH levels it caused an immediate reduction in the activity of the more concentrated solution. This result is best explained by some form of adsorption. Siminoff & Gottlieb (1951) have shown that streptomycin is bound by clay minerals; apparently carefully washed sand is also sufficiently acidic to bind the streptomycin anion. This adsorption appears to be most marked in acid solutions.

Streptomycin was also adsorbed on all the soils (Fig. 17), and this resulted in immediate and complete inactivation of the 40 units/ml. solution. In Wareham II and IV, once the adsorptive capacity of the soil was saturated, no further loss of activity took place. In the remaining soils the rate of inactivation, after the initial adsorption, was influenced by the presence of buffer. In Wareham I, streptomycin in water solution was inactivated slowly in sterile soil, rapidly in fresh soil; inactivation did not occur in fresh soil in buffer solution. A similar result was obtained in Wareham III, though here water solutions in fresh and partially sterile soils were inactivated equally rapidly. Inactivation was very rapid in garden soil, whether fresh or partially sterile.

Viridin

In buffer at pH 3·3 viridin (Fig. 18) is moderately stable; above pH 5·0 it is very rapidly inactivated. Detailed study of the data will show that in all cases inactivation follows a course equivalent to a first-order reaction and that the rate is directly related to the pH. Sand increases the rate of inactivation throughout the pH range tested. The stability of viridin in soil (Fig. 19) is primarily dependent on the pH of the soil. Inactivation is rapid in the neutral garden soil. Wareham II and IV accelerate inactivation to the same extent as sand; Wareham III has a rather more pronounced accelerating effect. In none of these soils is there any sign of biological inactivation. In fresh Wareham I soil, on the other hand, inactivation is much more rapid than in the same soil partially sterilized, in which inactivation is at the same rate as in sand.

CONCLUSIONS

These results show that the inactivation of antibiotics in soil may be the result of one or more distinct processes. These include: inactivation resulting from intrinsic instability of the antibiotic at the pH of the soil; inactivation as the result of microbiological activity; adsorption on some soil constituent, of which the clay colloids and humus particles are probably of greatest importance; some other kind of inactivation, probably chemical in nature, at present imperfectly understood.

The effect of soil pH on the rate of inactivation was most noticeable with the antibiotics albidin, frequentin, gliotoxin, penicillin and viridin.

Biological inactivation, expressed by more rapid inactivation in fresh than in partially sterile soil, occurred only in garden soil, Wareham I and Wareham III. These are the soils with the highest organic matter content and the most abundant microflora. Biological inactivation was most notable with the antibiotics griseofulvin, mycophenolic acid and patulin, but it was a contributory factor to the inactivation of several others.

Adsorption was an important factor in the inactivation of streptomycin solutions; it is noteworthy that carefully washed sand was capable of binding significant amounts of this basic antibiotic. The amount adsorbed was related to the pH of the solution. Adsorption was a less important factor in the inactivation of the acidic or neutral fungal antibiotics.

The rapid inactivation of gladiolic acid, penicillin and streptomycin in partially sterile garden soil, and some other similar observations, under pH conditions favourable to stability, suggests strongly that some other form of inactivation may occur. It is conceivable that the antibiotic in such cases is reacting chemically with a soil constituent, or that some soil constituent acts as a catalyst.

Finally, it may be noted that those antibiotics produced by fungi isolated from the acid Bagshot Sand soils were more stable in the acid Wareham series of soils than in the neutral garden soil. While no direct evidence is presented here that any of these antibiotics are produced naturally in soil, at least it may be said that all show a sufficient degree of stability, in some of the soils at least, for them to have a significant biological effect if produced.

My sincere thanks are due to Mr H. G. Hemming and his staff, for carrying out the large number of biological assays involved in this work, and to Boots Pure Drug Co. Ltd. for the gift of streptomycin.

REFERENCES

Brian, P. W. (1946). Production of gliotoxin by *Penicillium terlikowskii* Zal. *Trans. Brit. mycol. Soc.* **29**, 211.

Brian, P. W., Curtis, P. J. & Hemming, H. G. (1946). A substance causing abnormal development of fungal hyphae produced by *Penicillium janczewskii* Zal. Part I. *Trans. Brit. mycol. Soc.* **29**, 173.

Brian, P. W., Curtis, P. J. & Hemming, H. G. (1948). Gladiolic acid, an antibiotic produced by *Penicillium gladioli* McCull & Thom. *J. gen. Microbiol.* **2**, 341.

Brian, P. W. & Hemming, H. G. (1945). Gliotoxin, a fungistatic metabolic product of *Trichoderma viride*. *Ann. appl. Biol.* **32**, 214.

Brian, P. W., Hemming, H. G. & McGowan, J. C. (1945). Origin of a toxicity to mycorrhiza in Wareham Heath soil. *Nature, Lond.* **155**, 637.

Brian, P. W. & McGowan, J. C. (1945). Viridin: a highly fungistatic substance produced by *Trichoderma viride*. *Nature, Lond.* **156**, 144.

Curtis, P. J. & Grove, J. F. (1947). A fungistatic and bacteriostatic red pigment produced by a strain of the *Penicillium nigricans janczewskii* series. *Nature, Lond.* **160**, 574.

Curtis, P. J., Hemming, H. G. & Smith, W. K. (1951). Frequentin, an antibiotic produced by *Penicillium frequentans* Westling. *Nature, Lond.* **167**, 557.

Florey, H. W., Chain, E., Heatley, N. G., Jennings, M. A., Sanders, A. G., Abraham, E. P. & Florey, M. E. (1949). *Antibiotics.* Oxford University Press.

Foster, J. W. & Woodruff, H. B. (1944). Microbiological aspects of penicillin. VI. Procedure for cup assay for penicillin. *J. Bact.* **47**, 43.

Jefferys, E. G. & Smith, W. K. (1951). A new type of soil percolator. *Proc. Soc. appl. Bact.* **14**, 169.

Pramer, D. & Starkey, R. L. (1951). Decomposition of streptomycin. *Science*, **113**, 127.

Rayner, M. C. (1945). Origin of toxicity to fungi in Wareham Heath soil. *Nature, Lond.* **156**, 174.

Siminoff, P. & Gottlieb, D. (1951). The production and role of antibiotics in the soil. I. The fate of streptomycin. *Phytopathology*, **41**, 420.

Waksman, S. A. (1949). *Streptomycin, Nature and Practical Applications.* London: Baillière, Tindall and Cox.

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Susceptibility of Pleuropneumonia-like Organisms from Human Genital Tract to the Action of Soaps

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SUMMARY: Pleuropneumonia-like organisms (PPLO) of human origin are very susceptible to the deleterious action of soaps. There was no significant difference in the antibacterial action of soaps containing 2% hexachlorophene and an ordinary hand soap, on the pleuropneumonia-like organisms tested. The great susceptibility of these organisms to soap probably masks any action which the hexachlorophene contained in the medicated soaps might have on PPLO. The deleterious action of soaps on pleuropneumonia-like organisms is bactericidal.

The fragility of pleuropneumonia-like organisms (PPLO) from man and the L-forms of bacteria has been stressed for many years by Dienes and summarized in his recent review (Dienes & Weinberger, 1951). The best information concerning the nature of a cell wall or bordering membrane has been contributed by studies with the electron microscope as the small size of these organisms does not permit good cytological studies with the light microscope.

From electron microscope studies of bovine pleuropneumonia organisms and Seiffert's saprophytic organisms, Ruska & Poppe (1948) concluded that the bordering substance of the organisms should be considered as a plasmalemma rather than as a cell wall as encountered in bacteria. The covering substance does not seem to be different from the inner substance. It has a strong attachment to the inner substance, is plastic and changeable in form. The authors were unable to coagulate and remove the cytoplasm from the cell membrane by means of heat as it is possible to do with bacteria.

Smith, Hillier & Mudd (1948), in studying with the electron microscope two strains of PPLO isolated from man, observed that the round bodies and rod-shaped cells were readily destroyed by immersion in distilled water, which proved that they did not possess a sturdy outer structure comparable to the cell wall of ordinary bacteria. In one strain the rod forms had a limiting structure from which the cytoplasm appeared to separate upon drying. The size, shape, and cell-wall structure led these authors to conclude that the PPLO must be regarded as indubitable bacterial cells.

Since it may be assumed that the visible elements of PPLO are probably bacterial in nature but with a cell wall more fragile than that of ordinary bacteria, it is reasonable to suppose that the organisms would be very susceptible to agents which lower the surface tension as, for example, soap. The experimental work to be described bears out this supposition.

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EXPERIMENTAL

Cultures

Staphylococcus aureus, the F.D.A. strain customarily used for phenol coefficient testing. It was killed in exposures of 5, 10 and 15 min. by phenol, 1 : 70, when subcultured into extract broth or into fluid thioglycollate medium but it was not killed by phenol, 1 : 80, under the same conditions.

Bacterium coli, P3, a communis strain.

Pleuropneumonia-like organisms. All were of human origin: Strain 07P was received from I. G. Schaub, Johns Hopkins University, in 1948. The same strain, 07, and strain 09 were received from the same source in 1949. Strain 48 was isolated from urethral discharge, strain 60 from the cervical canal, and strains 43 and 107 were isolated by us from prostatic secretions.

Media

Extract broth had the following composition: beef extract (Difco), 0·3 %, peptone (Parke-Davis), 1 %, and sodium chloride, 0·5 %. The pH was 7·2 after sterilization in the autoclave.

Bacto-heart infusion broth enriched with Bacto-PPLO bovine serum fraction A, 1 % (Smith & Morton, 1951), was employed when it was necessary to grow PPLO in a liquid medium. For ascertaining growth of PPLO in liquid media it is necessary to subculture on to solid medium. For this purpose Bacto-PPLO agar (Morton, Smith & Leberman, 1951) enriched with Bacto-PPLO bovine serum fraction A was used. All cultures were incubated aerobically at 37°.

Soaps

Dial soap was purchased over the counter. It is a bar soap stated to contain 2 % hexachlorophene (G-11) and is manufactured by Armour and Co. Gamophen antiseptic soap was kindly supplied by Dr Herbert L. Davis, Ethicon Suture Labs. It is a bar soap stated to contain 2 % hexachlorophene. Ivory soap (manufactured by Proctor and Gamble), was employed as an example of non-medicated soap for a control. Solutions of the various soaps were prepared by dissolving 1 g. of thin shavings from the bars in sterile distilled water in a glass-stoppered graduated cylinder, usually diluting to a volume of 50 ml. The soap solutions were freshly prepared, and subsequent dilutions of soaps were prepared with sterile distilled water from these initial solutions.

Methods

For determining the antibacterial action of soaps, 5 ml. of the soap solutions were placed in sterile test-tubes, 16 × 150 mm. In the case of the bacterial cultures 0·5 ml. of a 24 hr. broth culture was added to each medication tube. In the case of the strains of PPLO a block of agar containing colonies of PPLO was placed in a tube of the enriched heart infusion broth and incubated for 3 days. One-half ml. amounts of these cultures were added to the medication tubes. The temperature of the medication was room temperature.

For subculturing, 0·5 ml. portions were transferred at stated intervals from the medication tubes to the subculture medium. In the case of the bacteria the subculture medium was 10 ml. amounts of extract broth. In the case of the primary subcultures of the bacteria from the medication tubes growth was ascertained by turbidity and Gram staining. Those tubes showing no growth were kept under observation for 1 week. In the case of the PPLO the subculture medium was 5 ml. amounts of the enriched heart infusion broth. These primary subcultures of PPLO were incubated for 3 days at 37° and then a loopful was streaked over the surface of the enriched PPLO agar. These cultures were incubated for 3 days and then examined for characteristic colonies by the microscope, employing a magnification of ×100. This step was necessary to determine whether growth had taken place in the primary subcultures, as growth of PPLO in liquid media cannot be determined by turbidimetric or staining procedures.

Because of the low surface tension of soap solutions and the wide range in concentration of the soap solutions used, as well as the desirability of employing a large number of test organisms where bacteriostatic action is being studied, subcultures were made by transferring 0·5 ml. amounts with sterile pipettes from the medication tubes. The possible advantages of this volumetric transfer have been discussed previously (Tobie & Orr, 1944; Tice & Pressman, 1945; Pressman & Rhodes, 1946; Morton, North & Engley, 1948).

To insure that the inoculum in the subculture tubes did not contain sufficient material to exert a bacteriostatic effect in the subcultures, the following control was made. One-half ml. of culture was added to 5 ml. of sterile distilled water. After 15 min. 0·5 ml. of the diluted culture was transferred to 10 ml. of extract broth containing 0·5 ml. of the respective soap solution.

RESULTS

The results obtained when the three bar soaps were tested against *Bact. coli* are summarized in Table 1. The three soaps did not sterilize the test culture. These results are in agreement with the findings of Walker (1925) who found that a white floating soap and a perfumed toilet soap did not kill *Bact. coli* when using the stiffest possible lather which was determined to be about 8% with the soaps used. However, certain chemically pure soaps have a killing action on *Bact. coli*. Walker (1925) reported that 0·2 N solutions of sodium

Table 1. *The effect of soaps on* Bacterium coli *when subcultures are made from medication tubes to extract broth*

	Time of exposure			
Soap	Control	5 min.	10 min.	15 min.
Ivory, 1 : 50	+	+	+	+
Dial, 1 : 25	+	+	+	+
Gamophen, 1 : 50	+	+	+	+

+ = growth of test organism.

Control = 0·5 ml. culture added to 5 ml. distilled water. After 15 min. 0·5 ml. transferred to 10 ml. extract broth containing 0·5 ml. of the respective soap solutions.

oleate and sodium linolate had no killing action, but 0·1 N solutions of sodium laurate and sodium palmitate killed. These results are comparable to the findings of Reichenbach (1908).

The results obtained when the three bar soaps were tested against *Staph. aureus* are summarized in Tables 2 and 3. The results with Ivory soap are comparable to those obtained earlier with other toilet and household soaps (Morton, 1944), namely, no killing action. In the case of the bacteriostatic control with Dial soap, the soap carried into the subculture medium with the inoculum did not inhibit the growth of the test organisms. However, failure to get growth in the subcultures from some of the medication tubes may have been due to the fact that the test organisms had been in intimate contact with the soap and were in a state of bacteriostasis.

Table 2. *The effect of soaps on* Staphylococcus aureus *when subcultures are made from the medication tubes into extract broth and when subcultures from the gamophen-containing tubes are made into extract broth containing* 10 % *serum.*

		Time of exposure		
Soap	Control	5 min.	10 min.	15 min.
	Subcultures made into extract broth			
Ivory, 1 : 50	+	+	+	+
Dial, 1 : 500	+	−	−	−
1 : 600	·	−	−	−
1 : 700	·	−	−	−
1 : 800	·	+	+	+
Gamophen, 1 : 200	−	−	−	−
1 : 400	−	−	−	−
1 : 800	−	−	−	−
1 : 1200	+	−	−	−
1 : 1600	+	−	−	−
	Subcultures made in extract broth plus 10 % serum			
Gamophen, 1 : 100	+	+	+	+
1 : 200	+	+	+	+
1 : 400	+	+	+	+

+ = growth; − = no growth of test organism.

Control = 0·5 ml. culture added to 5 ml. distilled water. After 15 min. 0·5 ml. transferred to 10 ml. of the subculture medium containing 0·5 ml. of the respective soap solution.

It was readily apparent from the control tubes that sufficient gamophen soap was carried into the subculture with the inoculum to inhibit viable organisms which might be present in the inoculum. It was necessary, therefore, to add a substance such as serum to the subculture medium to neutralize the bacteriostatic action of the soaps. It has been known for some time that serum will decrease the bacteriostatic and lytic action of soaps (Lamar, 1911; Walker, 1924). With 10 % bovine serum present in the subculture medium to neutralize the bacteriostatic action of the soaps, growth took place in all subculture tubes.

The presence of serum or of certain fractions of serum (Edward & Fitzgerald, 1951; Smith & Morton, 1951) appears to be necessary for the growth of PPLO *in vitro.* Since whole serum is frequently inhibitory to PPLO and PPLO bovine serum fraction A is free of the inhibiting substances, the latter substance was used for enriching the culture medium. It was necessary, therefore, to determine whether PPLO bovine serum fraction A, like whole serum, was capable of decreasing the bacteriostatic action of soap. For this purpose 0·5 ml. of a broth culture of *Staph. aureus* was added to each of four dilutions of Dial soap. After 10 min. exposure at room temperature, 0·1 ml. of the soap + culture mixture was transferred to (*a*) a tube of extract broth, (*b*) a tube of extract broth containing 10 % bovine serum, and (*c*) a tube of extract broth containing 2 % PPLO bovine serum fraction A. The results are summarized in Table 3; it can be seen that PPLO bovine serum fraction A and serum have a similar action in this respect.

Table 3. *Ability of serum and PPLO bovine serum fraction A to neutralize the bacteriostatic action of Dial soap on* Staphylococcus aureus

	Extract broth	Extract broth + 10 % serum	Extract broth + 2 % fraction A
Dial, 1 : 200	–*	+	+
1 : 400	–*	+	+
1 : 800	–†	+	+
1 : 1600	–†	+	+

– = no growth; + = growth of test organism.

* No growth obtained when one loopful of subculture was streaked on extract agar.

† Growth of test organism obtained when one loopful of culture was streaked on extract agar.

The results of the tests with PPLO against non-medicated and medicated soaps are given in Table 4. Because of the nature of PPLO two types of controls were run; a dilution control and a bacteriostatic control. One-half ml. of culture of PPLO was added to 5 ml. of sterile distilled water. After 10 min. 0·5 ml. was transferred to 5 ml. of liquid medium for the dilution control. After these subcultures were incubated for 3 days, one loopful from each was streaked on solid medium. In every case growth of the test organism took place. After the 10 min. exposure to distilled water 0·5 ml. of the diluted culture was transferred to 5 ml. of liquid medium containing 0·5 ml. of 1 : 1000 dilution of the respective soap for a bacteriostasis control. After these subcultures were incubated for 3 days, one loopful from each was streaked on solid medium. In the majority of instances the test organism grew. The results of this control are incorporated in Table 4.

DISCUSSION

The presence of serum or a fraction of serum in the culture medium necessary for the growth of PPLO also neutralizes the bacteriostatic action of soap and hexachlorophene. Failure to get growth of these organisms in the subcultures indicated that the action of soaps was bactericidal.

Table 4. *The effect of soaps on pleuropneumonia-like organisms*

		Ivory soap			Dial soap		
Culture	Dilution	Control*	5 min.	10 min.	Control	5 min.	10 min.
PPLO, strain 07P	1 : 1,000	+	−	−	+	−	−
	1 : 2,500	.	−	−	.	−	−
	1 : 5,000	.	+	+	.	+	+
	1 : 10,000	.	+	+	.	+	+
PPLO, strain 09	1 : 1,000	+†	−	−	+	−	−
	1 : 2,500	.	−	−	.	−	−
	1 : 5,000	.	−	−	.	−	−
	1 : 10,000	.	+	+	.	+	+
PPLO, strain 43	1 : 1,000	+	−	−	+	−	−
	1 : 2,500	.	−	−	.	−	−
	1 : 5,000	.	−	−	.	+	−
	1 : 10,000	.	+	+	.	+	+
PPLO, strain 07	1 : 1,000	+	−	−	−	−	−
	1 : 2,500	.	−	−	.	−	−
	1 : 5,000	.	+	+	.	+	−
	1 : 10,000	.	+	+	.	+	+
PPLO, strain 107	1 : 2,500	+	.	−	.	.	.
	1 : 5,000	.	.	+	.	.	.
	1 : 10,000	.	.	+	.	.	.
PPLO, strain 48	1 : 1,000	−	−	−	±	−	−
	1 : 2,500	.	−	−	.	−	−
	1 : 5,000	.	−	−	.	−	−
	1 : 10,000	.	+	+	.	+	+
PPLO, strain 60	1 : 1,000	±	−	−	+	−	−
	1 : 2,500	.	−	−	.	−	−
	1 : 5,000	.	+	+	.	−	−
	1 : 10,000	.	+	+	.	+	−

− = no growth in subcultures; + = growth in subcultures; ± = growth in one experiment, no growth in another experiment.

* Control for bacteriostasis: 0·5 ml. culture added to 5 ml. distilled water. After 10 min. (*a*) 0·5 ml. transferred to 5 ml. liquid culture medium containing 0·5 ml. 1 : 1,000 dilution of the respective soaps; (*b*) 0·5 ml. transferred to 5 ml. liquid medium. After incubation for 3 days one loopful from each tube was streaked on solid medium to determine if growth of the organisms had taken place. In every case the dilution controls (*b*) were positive. The results of (*a*) the bacteriostasis controls are given in the table.

† Culture contaminated with bacteria.

It has been found that *Haemophilus influenzae*, *Streptococcus pneumoniae* (Nichols, 1920), *Corynebacterium diphtheriae* (Walker, 1925), *Neisseria meningitidis*, *N. gonorrhoeae* (Walker, 1926), and *Treponema pallidum* (Reasoner, 1917) are readily destroyed by ordinary soaps. *Strep. pyogenes* (Nichols, 1920; Walker, 1931) and *Salmonella typhi* (Walker, 1925) are also destroyed by soaps but less readily.

The susceptibility of PPLO to the action of soaps is much greater than that of the highly susceptible bacteria. It is interesting to note that Warren (1942) observed that the susceptibility of pleuropneumonia organisms and certain L-forms to hydrogen peroxide, phenol, and ultraviolet light was of the same magnitude as that of some of the ordinary bacteria. The unusually high susceptibility of PPLO to soaps is probably due to the fact that the organisms possess a very fragile cell structure.

REFERENCES

DIENES, L. & WEINBERGER, H. J. (1951). The L-forms of bacteria. *Bact. Rev.* **15**, 245.

EDWARD, D. G. FF. & FITZGERALD, W. A. (1951). Cholesterol in the growth of organisms of the pleuropneumonia group. *J. gen. Microbiol.* **5**, 576.

LAMAR, R. V. (1911). Chemo-immunological studies on localized infections. Second paper: Lysis of the pneumococcus and hemolysis by certain fatty acids and their alkali soaps. *J. exp. Med.* **13**, 380.

MORTON, H. E. (1944). 'Germicidal' soaps. I. The importance of a clean skin, the action of soaps in freeing the skin of viable microorganisms, and methods for testing the efficiency of germicidal (medicated) soaps. *J. Amer. med. Ass.* **124**, 1195.

MORTON, H. E., NORTH, L. L. & ENGLEY JR., F. B. (1948). The bacteriostatic and bactericidal actions of some mercurial compounds on hemolytic streptococci. *J. Amer. med. Ass.* **136**, 36.

MORTON, H. E., SMITH, P. F. & LEBERMAN, P. R. (1951). The cultivation of pleuropneumonia-like organisms from the human genitourinary tract with reference to their possible venereal transmission. *Amer. J. Syph.* **35**, 14.

NICHOLS, H. J. (1920). Bacteriologic data on the epidemiology of respiratory diseases in the Army. *J. Lab. clin. med.* **5**, 502.

PRESSMAN, R. & RHODES, J. C. (1946). Sources of error in germicidal activity tests with quaternary ammonium compounds. *Soap sanit. Chemicals*, **22**, 137, 139, 141, 143.

REASONER, M. A. (1917). The effect of soap on *Treponema pallidum*. *J. Amer. med. Ass.* **68**, 973.

REICHENBACH, H. (1908). Die desinfizierenden Bestandteile der Seifen. *Z. Hyg. InfektKr.* **59**, 296.

RUSKA, H. & POPPE, K. (1948). Elektronenmikroskopische Untersuchungen zur Morphologie der Seiffertschen Mikroorganismen und des Erregers der Lungensuche des Rindes. *Z. Hyg. InfektKr.* **127**, 201.

SMITH, P. F. & MORTON, H. E. (1951). The separation and characterization of the growth factor in serum and ascitic fluid which is required by certain pleuropneumonia-like organisms. *J. Bact.* **61**, 395.

SMITH, W. E., HILLIER, J. & MUDD, S. (1948). Electron micrograph studies of two strains of pleuropneumonia-like (L) organisms of human derivation. *J. Bact.* **56**, 589.

TICE, L. F. & PRESSMAN, R. (1945). Antiseptics of the quaternary ammonium type in the presence of positive and negative gelatin. *J. Amer. pharm. Ass.* (scientific ed.), **34**, 201.

TOBIE, W. C. & ORR, M. L. (1944). Determination of phenol coefficients in presence of surface tension depressants. *J. Lab. clin. Med.* **29**, 767.

WALKER, J. E. (1924). The germicidal properties of chemically pure soaps. *J. infect. Dis.* **35**, 557.

WALKER, J. E. (1925). The germicidal properties of soap. *J. infect. Dis.* **37**, 181.

WALKER, J. E. (1926). The germicidal properties of soap. *J. infect. Dis.* **38**, 127.

WALKER, J. E. (1931). The germicidal and therapeutic applications of soaps. *J. Amer. med. Ass.* **97**, 19.

WARREN, J. (1942). Observations on some biological characteristics of organisms of the pleuropneumonia group. *J. Bact.* **43**, 211.

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Variation in the Antigenic Composition of Staphylococcal Coagulase

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SUMMARY: Four different antigenic types of coagulase were identified. Antisera were prepared in rabbits against three of these, and a specific antiserum against the fourth was found in a normal human. The coagulases of all but two of the human strains examined were inhibited by one or other of two of these antisera and in a few cases by both; in addition antibodies of the same type were present in the sera of the majority of a group of normal adults. The coagulases produced by thirty-five animal strains were inhibited by the other two antisera, but further work is needed on the coagulases found in animal strains.

Several workers (Lominski & Roberts, 1946; Bekker, 1947; Tager & Hales, 1948; Rammelkamp, Hezebicks & Dingle, 1950) have described antibodies to staphylococcal coagulase in normal sera and plasma. By cross-neutralization tests with the sera of a group of 148 normal individuals against the coagulases of different staphylococcal strains, Rammelkamp *et al.* (1950) found that these coagulases fell into three different antigenic types. Many sera inhibited more than one of the three coagulases finally selected for intensive study, but there was usually a wide variation between the titres obtained in each serum, and some sera were almost specific for an individual coagulase. Immunization experiments in monkeys showed that two of the coagulases isolated were very nearly distinct antigenically, but the third type appeared to be related to the other two.

The development of a simple and reliable method for the production of antibodies in rabbits against coagulase (Duthie & Lorenz, 1952) made it possible to investigate these claims more thoroughly. The present paper reports this work, together with a limited survey of the antibodies to staphylococcal coagulase and α-toxin present in human sera from a small group of laboratory workers.

METHODS

Strains. For the isolation of good coagulase-producing colonies, strains were plated out on nutrient agar containing sterile fibrinogen and human plasma. Strains which did not show a zone of clotted fibrin around each colony were not suitable for study, although such strains might give a positive slide test with plasma. Suitable colonies were either grown immediately in the fluid medium or were stored as stab cultures in nutrient agar at 4°.

Preparation and assay of coagulase for antigenic typing. Cultures were made in 25 ml. conical flasks containing 2 ml. of digest broth supplemented by 0·3 % bovine albumin (Armour). They were shaken on a tilted gramophone turntable at 37° until fully grown, when the supernatants were separated by centrifuging. For assay 0·1 ml. of falling dilutions of the supernatants, usually at 1/5, 1/10, 1/30 and 1/90 in veronal buffer at pH 7·0, were mixed with 0·1 ml. of normal rabbit serum, diluted to 1/5, in tubes 5 × 0·6 mm. After 30 min. on the bench 0·2 ml. of a mixture of 0·1 % (w/v) bovine or human fibrinogen and fresh rabbit plasma 0·5 % (v/v) was added to each tube and the rack was incubated for 1 hr. at 37°. One drop of a 5 % (w/v) suspension of 'Filter-cel' (Johns-Manville) was added to each tube and, after shaking, the rack was placed for a short time in the refrigerator before reading. Tubes might show a clot, a broken clot, gross floccules, finer floccules, or floccules just distinguishable from those in the control tubes. The titre of the coagulase chosen for use in the final test was taken as that giving either a broken clot or gross floccules and sometimes both strengths were used; these values could be readily determined with practice.

Antigenic typing of coagulase. In this test each coagulase was at that dilution which in the preliminary titration gave either a broken clot or gross floccules i.e. at about 10–20 M.C.D./ml. (M.C.D. defined below) Antisera and serum controls were diluted 1/5 and mixed with the coagulases at the levels chosen and held for 30 min. at room temperature. An equal volume of the fibrinogen + plasma mixture was then added and after 1 hr. at 37° 'Filter-cel' added and the tubes read as before.

Purified coagulases were stable during the test period, but many fresh preparations contained the coagulase in an unstable form; although the supernatants had been stored in the cold, the titres varied between the preliminary titration and typing. In such cases assays were repeated and correspondingly lower dilutions used.

Preparation of antisera. This was essentially as described in the previous work (Duthie & Lorenz, 1952). The subcutaneous injection at fortnightly intervals of 10 mg. of partially purified high titre coagulase adsorbed on 3 mg. $AlPO_4$, gave relatively good titres in 8–10 weeks, which could be maintained or even raised by subsequent injections.

Antibody assay against purified coagulase. This was as previously described (Duthie & Lorenz, 1952) except that veronal buffer was used as a diluent. Sera were not inactivated, but heparin 2–5 units/ml. was added after separation, and the sera held at 37° to destroy thrombin. The mixtures consisted of 0·1 ml. of falling dilutions of serum and 0·1 ml. of coagulase (20 M.C.D./ml.) which were left at room temperature for 30 min. after which 0·2 ml. of the fibrinogen-plasma mixture was added and the tubes incubated for 1 hr. at 37°. One drop of 5 % 'Filter-cel' was added to each tube before reading. The reciprocal of the serum dilution just sufficient to allow minimal clotting was taken as the titre of the serum expressed in units. The M.C.D. or minimal clotting dose of the purified coagulase was that amount of coagulase/ml. which, when mixed with 1 ml. of plasma fibrinogen and incubated at 37° for 1 hr., gave minimal flocculation after the addition of one drop of 'Filter-cel' to 0·4 ml. of the mixture. This standard is variable since it depends on the stability of a particular fibrinogen preparation; in practice certain sera were stored frozen and the standard coagulases were kept as lyophil dried powders. α-Antitoxin was determined at the 1/10 unit level in the usual manner.

RESULTS

A preliminary survey of some fifty strains from human lesions showed that with few exceptions the coagulases were completely inhibited by an antiserum, diluted 1/5, prepared against the 'Newman' strain coagulase previously described. The exceptions were in part inhibited by the antiserum, and a reduction in the concentration of the coagulase below the level normally used gave complete inhibition. At this stage ten strains of animal origin were received from Mr Proom, and it was found that many of these produced a coagulase which was not inhibited by the 'Newman' antiserum. At the same time strain 104 was received from Dr Tager, and since its coagulase was not inhibited it was decided to immunize rabbits with coagulase from strain 104 and from one of Mr Proom's strains, CN 59. For clarity the coagulase from the 'Newman' strain (human origin) will be referred to as coagulase A, that from CN 59 (bovine origin) as B, and that from Tager's 104 (human origin) as C. The corresponding antisera are named antiserum A, B and C.

All these strains produced a stable coagulase in high titre and it was possible to purify the material by precipitation with $CdSO_4$ followed by dialysis against 0·01 N-HCl (Duthie & Lorenz, 1952). Injection of this material into rabbits gave good titres and cross-neutralization tests, shown by Table 1, were reasonably specific. In the final stages of the study it was found that a number of staphylococcal strains, freshly isolated from superficial infections in humans, produced a fourth coagulase which was not completely inhibited by any of the antisera A, B and C, and only in part by antiserum A. Attempts to prepare an antiserum in rabbits were unsuccessful, but as the majority of human sera contained at least five units of antibody to this coagulase/ml. there was little difficulty in finding one which had 100 units/ml. with little or none against any of the other coagulases. This serum, antiserum D, also appears in Table 1.

Table 1. *Cross-neutralization of coagulases A, B, C and D by their respective antisera*

	Antiserum											
	A			B			C			D		
	Serum dilutions											
Coagulase	5	10	20	5	10	20	5	10	20	5	10	20
A	–	–	–	++	+++	BC	BC	BC	BC	BC	BC	BC
B	BC	BC	BC	–	–	–	C	C	C	C	C	C
C	++	+++	BC	+++	+++	+++	–	–	–	BC	BC	BC
D	+++	+++	BC	BC	BC	BC	BC	BC	BC	–	–	–

C = clot; BC = broken clot; +++ and ++ = larger and smaller floccules; – = no floccules.

All sera, including the human serum D, had titres of between 60 and 100 units/ml. and at the dilution of 1/5 employed in typing other coagulases there was good specificity for the four antigens together with a large excess of antibody. Sera were stored frozen, since a preservative could not be added without risk of harming the coagulase in the test. Under these conditions there was some deterioration in potency, which was never more than *c.* 30 % in several months.

Antigenic pattern of the coagulase from different strains

Apart from the coagulases first tested with antiserum A, the coagulases produced by eighty-five strains from infections in fifty humans and thirty-five animals (cows and sheep) were tested with these antisera with the results shown in Table 2. The fifty human strains are probably representative, since they were in all but one case, i.e. Tager's strain 104, chosen from a random series of staphylococcal infections, mostly of a superficial nature, encountered in hospital practice. Coagulases of five strains were inhibited equally by two different antisera, three by A and C and two by A and D. Six were completely inhibited only by antiserum D and partly inhibited by A, while the remainder were inhibited by antiserum A. Thus some 86 % of these human strains produced a coagulase which was inhibited by antibodies to coagulase A.

Table 2. *Inhibition of the coagulases produced by eighty-five staphylococcal strains of human and animal origin*

	Origin of strains inhibited by		
Antisera	Man	Cow	Sheep
A	38	11	1
B	—	2	1
C	1	5	10
D	6	—	—
A and C	3	5	—
A and D	2	—	—
Totals	50	23	12

The thirty-five animal strains were from three sources. Fifteen strains isolated locally by Mr J. C. Francis from cases of bovine mastitis all produced coagulases which were inhibited by antiserum A and four were in addition inhibited by antiserum C. The remaining twenty were stock laboratory strains received from Mr Foggie and Mr Proom. One of these, a strain of ovine origin, produced a coagulase only inhibited by antiserum A; while another of bovine origin produced a coagulase inhibited by both A and C antisera. The coagulases produced by the remaining eighteen strains (bovine and ovine) were inhibited by antisera B or C. Apart from these eighty-five strains there were four, two of human origin and two of bovine origin, which were partially inhibited only by antiserum A, and which in consequence were untypable.

Antibodies in normal human sera

Since a previous survey (Duthie & Lorenz, 1952) showed that antibodies against type A coagulase were present in the sera of the majority of a group of twenty-five laboratory workers, it was decided to repeat these tests measuring in addition antibodies against B, C and D type coagulases and against α-toxin. Although six of the twenty-five sera now tested were from individuals other than those measured 6 months previously, the distribution of the anti-A coagulase levels was very similar (Table 3). Thus ten had between 0 and 1 unit/ml., eleven had 2–10 units/ml. and four had 15–80 units/ml. The nineteen persons measured previously showed little or no change in their serum levels except at the 0–1 unit level which is difficult to read and which has shown discrepancies in the past, either because of residual traces of thrombin or because of the instability of coagulase in unbuffered and undiluted serum. The addition of 2 M-KH_2PO_4 to a final concentration of about 0·04 M has greatly improved the consistency of these low-level readings.

Table 3. *Estimation of anticoagulase and α-antitoxin titres in units/ml. in the sera of twenty-five laboratory workers*

	Anticoagulase			
Serum no.	Anti-A	Anti-C	Anti-D	α-Antitoxin
1	1	0	7	0·8
2	2	1	2·5	0
3	30	20	0	1·0
4	10	10	50	1·5
5	5	0	10	1·0
6	2·5	5	5	1·6
7	5	0	15	1·0
8	2	0	3	0·5
9	15	0	50	1·5
10	10	0	10	2·0
11	15	10	80	0·5
12	1	0	10	0
13	2	0	120	0·4
14	0	5	1	0·4
15	1	0	1	0·5
16	1	10	1	0·5
17	0	7	0	0
18	10	15	25	0·5
19	2	0	5	1·6
20	1	0	15	4·0
21	0	2	2	0·5
22	1	2	7	0·5
23	1	2	15	0·4
24	80	10	40	1·0
25	2	0	15	0

Only one of the twenty-five sera contained antibody against coagulase B, and as this antigen does not appear to be present in the coagulases produced by human strains, antibody B has not been included in Table 3. The antibodies varied widely between the sera, there being no relation between the levels of the three antibodies in different sera, nor did any anticoagulase bear any relation to the α-antitoxin level.

DISCUSSION

Four antigenically distinct types of coagulase have been encountered and it is likely that further search would reveal others, since the relatively crude method used in these experiments was intended to obscure possible subgroups. The existence of strains whose coagulase is inhibited by each of two antisera such as A and C or A and D suggests a common antigenic pattern to A and C and A and D which is hidden by other and more specific antigens, and this is borne out by Table 1 in which antigens C and D are partly inhibited by antiserum A.

More important is the finding that the coagulase of practically all the human strains examined is inhibited by antiserum A or D and mainly by the former. The high antibody titre of many human sera against these two antigens is possibly due to previous staphylococcal infections, though we have as yet no proof that these antibodies play any part in resistance. Of the twenty-five normal sera examined three giving the lowest titres against coagulase A came from the only individuals in the group who had suffered from staphylococcal infections in the past 12 months. Sera from a number of individuals with recurrent staphylococcal infections have in general had low antibody titres, but there have been two exceptions to this, and immunization experiments now in progress may help to decide if raising the antibody level in this type of infection is of value.

The position of the other antigens B and C is more doubtful since, apart from Tager's 104 strain of human origin, the only coagulases found which were inhibited only by either antiserum B or C, have come from stock strains of animal origin. The freshly isolated bovine strains examined produced coagulase which was completely inhibited by antiserum A and in part by antiserum C, as did some human strains, and it is not impossible that cultural conditions might change such strains into those producing coagulase C only. Until a large number of freshly isolated animal strains can be examined the role of these antigens in infection must remain in doubt.

It has unfortunately not been possible to compare the coagulase types found with those used by Rammelkamp *et al.* (1950), and it is always possible that they do not correspond with those used in the present studies. Each of the four types of coagulase found may consist of a number of different antigens, some of which are common to other coagulases. If this is the case then the classification which emerges in any study will be determined by the antigenic pattern of the first member studied. One important result of these studies is to provide two master strains and hence two master sera for the majority of staphylococcal coagulases, and it is hoped to use these for the investigation of the prevention of recurring staphylococcal infections by active immunization with the human type coagulases A and D.

My thanks are due to Dr C. H. Lack, Mr H. Proom, Dr M. Tager and Mr A. Foggie for staphylococcal strains. In addition, I wish to thank Mr J. C. Francis for animal strains and sera, the laboratory staff of the hospital for providing me with their sera, and Miss Sheila Isherwood for her valuable help. The work was made possible by a research grant from the South West Metropolitan Regional Hospital Board.

REFERENCES

Bekker, J. H. (1947). Studies on staphylocoagulase. II. Antistaphylocoagulase in human serum. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **13**, 128.

Duthie, E. S. & Lorenz, L. (1952). Staphylococcal coagulase: mode of action and antigenicity. *J. gen. Microbiol.* **6**, 95.

Hale, J. H. & Smith, W. (1945). The influence of coagulase on the phagocytosis of staphylococci. *Brit. J. exp. Path.* **26**, 209.

Lominski, I. & Roberts, G. B. (1946). A substance in human serum inhibiting staphylocoagulase. *J. Path. Bact.* **58**, 187.

Rammelkamp, C. H., Hezebicks, M. M. & Dingle, J. H. (1950). Specific coagulases of *Staphylococcus aureus*. *J. exp. Med.* **91**, 295.

Smith, W., Hale, J. H. & Smith, M. M. (1947). The role of coagulase in staphylococcal infections. *Brit. J. exp. Path.* **28**, 57.

Tager, M. & Hales, H. B. (1948). Differences in the resistance of human plasmas to staphylocoagulase. *Yale J. Biol. Med.* **21**, 91.

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Adaptation of 'B' Virus to the Swiss Albino Mouse

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SUMMARY: The 'B' virus (strain no. 1) of the 68th rabbit passage has been successfully transmitted to Swiss albino mice and carried through ten passages in this species by intracerebral inoculation. This virus produced symptoms of irritability and involvement of the central nervous system in these mammals. Paralysis occurred in mice from the 6th to the 10th passages. Virus-bearing material from the 10th intracerebral mouse brain passage upon being injected intradermally into unvaccinated rabbits produced paralysis but had no effect on rabbits previously immunized against 'B' virus.

The 'B' virus (strain no. 1) was isolated from the spleen and central nervous system of patient B and maintained since the original isolation in 1932 by serial passage in rabbits. This virus is extremely pathogenic for rabbits by most routes of inoculation. Following intracutaneous injection, a haemorrhagic necrotic area develops around the site of inoculation. About 1–2 days before paralysis occurs, most rabbits exhibit a tendency to bite the local lesion. Previous workers have found that the virus killed mice of about 2 weeks of age following intracerebral inoculation, but further serial passage in young mice was practically impossible. Primary intracerebral inoculation in mice of 3 weeks of age or older yielded irregular results and passage was also impossible (Sabin & Wright, 1934; Sabin, 1934). The present study was undertaken in an attempt to adapt this virus strain to Swiss albino mice (Webster & Dawson, 1935) so that this smaller mammal could be used for virus studies.

The 'B' virus (strain no. 1) used for this study was furnished by the American Type Culture Collection of Washington, D.C. Before receipt at our laboratory the virus had been passed dermally 67 times in rabbits. Lyophilized rabbit spinal cord of the 67th passage was received at this virus laboratory, ground with alundum and diluted to a 10% suspension with physiological saline.

EXPERIMENTAL

Three albino rabbits (weight 2 lb.) were inoculated intradermally with 0·1 ml. of this 10% suspension. The rabbits became paralysed in 7 days. When symptoms appeared the rabbits were sacrificed, and the spinal cords were removed aseptically. The pool of these infected rabbit spinal cords was ground with alundum and diluted to a 10% suspension with physiological saline. The suspension was then subjected to 5 min. centrifugation in an angle centrifuge at 2000 r.p.m. The supernatant was used to initiate the following experiment.

Three hundredths ml. of this 10% virus-bearing rabbit cord suspension was injected intracerebrally into each of six 21-day-old female Swiss albino mice (Webster Carworth strain). In mice responding to the injection of virus, irritability and malaise appeared in 12–14 days. In most cases these symptoms were followed by extreme nervousness, but there were no signs of paralysis until the 6th passage. Brains from mice sacrificed upon appearance of nervous symptoms were ground with alundum and diluted to a 20% suspension with physiological saline. Three hundredths ml. of this brain suspension was injected intracerebrally into 21-day-old white mice. Serial passages were continued through the 10th passage. The results of this experiment are recorded in Table 1. The inoculum for each passage consisted of 0·03 ml. of 20% brain suspension prepared from infected mice of the preceding passage.

Table 1. *Mouse passage of a strain of 'B' virus* (*strain no.* 1)

Passage no.	No. animals inoculated	No. animals paralysed, moribund or dead	No. days after inoculation nervous symptoms appeared
1	6	2	12–14
2	12	6	10–14
3	12	8	9–11
4	12	10	9–10
5	6	6	6–7
6	20	20	6–8
7	12	10	6–8
8	10	10	6–7
9	10	10	5–6
10	10	10	5–6

Virus-bearing mouse brain suspension from the 10th intracerebral passage was injected intradermally into three unvaccinated rabbits and into three rabbits which had previously been immunized with infected material from the 67th rabbit passage. High dilutions of virus were used to immunize these rabbits. After 6 days the unvaccinated rabbits showed paralysis, while the immunized group showed no nervous symptoms. This confirmed the virus from the 10th intracerebral mouse serial passage to the 'B' virus.

REFERENCES

Sabin, A. B. (1934). Studies on the 'B' virus. II. Properties of the virus and pathogenesis of the experimental disease in rabbits. *Brit. J. exp. Path.* **15**, 268.

Sabin, A. B. & Wright, A. M. (1934). Acute ascending myelitis following a monkey bite, with the isolation of a virus capable of reproducing the disease. *J. exp. Med.* **59**, 115.

Webster, L. T. & Dawson Jr., J. R. (1935). Early diagnosis of rabies by mouse inoculation. Measurement of humoral immunity to rabies by mouse protection test. *Proc. Soc. exp. Biol., N.Y.* **32**, 570.

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Oliver, W. H. (1952). *J. gen. Microbiol.* 7, 329–334.

The Stability of some Bacterial Enzymes toward Heat and Chemical Bactericides

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SUMMARY: The effect of heat, $HgCl_2$ and cetrimide on decarboxylase systems of some typical lactic acid bacteria and coliform organisms has been investigated. The enzymes appeared to be reversibly inactivated by heat, for the cultures in milk and in broth retained considerable decarboxylase activity after having been heated to 100° for 60 min. Furthermore, decarboxylation continued after sterilization of the cultures with $HgCl_2$ and with cetrimide.

It is well known that some enzymes even in an impure state can withstand temperatures considerably higher than that usually considered to cause irreversible denaturation of the protein moiety. Mellanby & Woolley (1913), Van Slyke & Cullen (1914), Stapp (1924), Bodansky (1924) and Northrop (1932) reported examples of stability of enzymes in the temperature range 80–100°. Miyake & Ito (1924) reported a diastase in *Aspergillus oryzae* which, although inactivated in a few seconds at 140°, required 130° for 15 min. and 120° for 60 min. for destruction. These appear to be the highest recorded temperatures at which any activity is retained by crude enzyme preparations. In spite of these early observations no one seems to have considered the possibility that bacterial enzymes might withstand the heat treatment commonly applied in pasteurization processes. The present work developed from the discovery of a heat-stable decarboxylase system in a strain of *Streptococcus diacetilactis*. The results show that bacterial enzyme systems may survive the killing of the organism by heat or bactericides and may continue under suitable conditions to produce characteristic end-products such as carbon dioxide and diacetyl.

MATERIALS AND METHODS

Test organisms. Four cultures of lactic streptococci were used. Two (DL and US) were strains of *Strep. diacetilactis*, a variant of *Strep. cremoris* with the unusual property of producing carbon dioxide, diacetyl and acetic acid in addition to the main-end product, lactic acid. This organism was first described by Matuzewski, Pijanowski & Supińska (1937), and characterized in more detail by Thiel (1940). The third culture was a strain of *Strep. cremoris* (HP), and the fourth (MX 13) was a dairy 'starter' containing a mixed flora of strains of *Strep. lactis*, *Strep. cremoris* and betacocci. The stock cultures were maintained in daily subculture in sterilized skim milk at 22°. All these cultures clotted milk within 24 hr. at 22° and gave a maximum acid concentration of *c.* 0·8 % (calculated as lactic acid) within 36 hr.

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Four strains of coliform organisms were used. According to the classification proposed by Wilson (1935) they were culture no. 5, *Bacterium aerogenes*, Type I; no. 13, *Bacterium coli*, Type II; no. 53, Intermediate, Type II; no. 83, *Bact. aerogenes*, Type II. All four strains produced a considerable amount of CO_2 in milk and in broth. Strains, nos. 13, 53 and 83, continued to produce gas during incubation for 12 days at 37° and strain 5 for 20 days. All the strains except no. 53 clotted skim milk at 37° within 7 days. The stock cultures were maintained in daily subculture in sterilized skim milk at 37°.

Media. (i) Skim milk sterilized in the autoclave at 12 lb. for 20 min. (ii) A broth containing 2·0 % lactose, 0·2 % yeast extract (Oxoid), 1·0 % Lab. Lemco, 1·0 % peptone, 0·8 % Na_2HPO_4 and sodium citrate sterilized as in (i).

Estimation of carbon dioxide. The CO_2 produced during growth of a culture was estimated by absorption in 0·1 N-baryta which was subsequently back-titrated with 0·1 N-oxalic acid, the amount of CO_2 being expressed in ml. at N.T.P. The culture vessels were 150 ml. conical flasks containing 100 ml. of culture medium connected to absorption flasks also of 150 ml. capacity.

Estimation of diacetyl. The diacetyl produced was estimated colorimetrically by the method of Pien, Baisse & Martin (1936) as modified by Cox & Wiley (1939).

RESULTS

In preliminary work the practice was to inoculate twenty-one flasks of medium with the organism under test, connect the flasks to absorption vessels containing baryta, incubate at 22°, and estimate the residual baryta in three flasks on each of seven successive days. The average of the three values obtained on each day was used to calculate the aggregate amount of CO_2 formed by the culture up to that time. Later the procedure was simplified by detaching the absorption flasks each day for titration and replacing them immediately with flasks containing fresh baryta. In this way the CO_2 production of the same three or four cultures was followed for the whole experimental period, with the advantage that possible variations from one culture flask to another were greatly reduced. This variation in procedure did not affect total CO_2 production, although there was a tendency for the end-point to be reached a little earlier since the admission of oxygen to the system apparently influences slightly the rate of reaction but not the position of the end-point. Table 1 shows the cumulative volumes (at N.T.P.) of CO_2 produced by the four cultures of lactic streptococci over a 7-day period. Culture HP, a typical *Strep. cremoris*, produced a negligible amount of CO_2. The other cultures produced CO_2 continuously during the 7-day period, the total volume produced being similar for all three. All the cultures clotted the milk medium within the first 24 hr. and acid production was negligible from then on. The numbers of lactic acid bacteria in clotted milk cultures are known to be static and thus it is evident that continued CO_2 production after the first 24 hr. under these conditions must be due to the metabolic activity of non-proliferating (so-called 'resting') bacterial cells.

Table 1. *Carbon dioxide produced by 100 ml. cultures of lactic streptococci in milk at 22°*

Days ...	1	2	3	4	5	6	7
	CO₂ produced (ml. N.T.P.)						
Culture							
US	11	21	29	34	38	40	43
DL	10	20	28	33	37	39	42
HP	1·5	2	3	4	4·5	5	5
MX 13	8	22	32	38	42	45	48

Controls of 100 ml. sterilized milk produced *c.* 2 ml. CO_2 during the 7-day period.

Carbon dioxide production after death of the bacteria

After the CO_2-producing characteristics of the cultures had been established, experiments were set up in which some of the cultures, after growth under the described experimental conditions for various times, were treated in the following ways: (i) heated to 80° for 2 min. and rapidly cooled; (ii) heated to 100° momentarily and rapidly cooled; (iii) treated with $HgCl_2$ at a concentration of 0·2 % w/v in the medium; (iv) treated with cetrimide 1 % w/v (cetyltrimethylammonium bromide). Incubation and daily estimation of CO_2 was continued with these flasks as with the controls for 7 days.

Table 2. *Carbon dioxide produced by 100 ml. milk cultures of* Strep. diacetilactis *in 7 days at 22°, the organisms being killed at various times from the start of the experiment.*

Killed after (days): ...	1	2	3	4
	CO₂ produced (ml. N.T.P.)			
Method of killing				
Heated at 80°, 5 min.	44	41	43	40
Heated at 100° momentarily	42	43	42	44
$HgCl_2$, 0·2 % (w/v)	40	43	44	42
Cetrimide 1·0 % (w/v)	40	39	41	41

Controls (viable organisms) produced 42–43 ml. during the same period.

Table 2 gives some typical results with culture DL. It will be observed that the various treatments had no significant effect on the total volume of CO_2 produced in 7 days. Similar results were obtained with all four cultures of lactic streptococci. The 'killed' cultures were tested for viable organisms after subjection to the various treatments by subculture into sterilized skim milk, and where $HgCl_2$ had been used, Na_2S was added in slight excess to eliminate the effect of mercury ions transferred with the inoculum. All four treatments were found to have sterilized the cultures. It was thus clear that the killed lactic streptococci contained an enzyme system capable of producing CO_2 in volumes similar to those produced by the viable bacteria.

Thermal destruction point of the bacterial enzymes

Preliminary trials indicated that the CO_2-producing enzyme system of the lactic streptococci retained considerable activity after being heated at 100° for 1 hr. in milk but that it was less stable in broth. A more precise heating technique permitted more detailed observations to be made. The flasks containing 100 ml. of culture and connected to CO_2-absorption flasks were plunged into a large water-bath maintained at about 2° above the desired temperature of treatment and agitated frequently. The time required for the culture fluid to reach the required temperature was checked by means of a thermometer in a control flask in the bath. After immersion for the desired time the flasks were removed from the bath and cooled in ice-water. In practice not more than 3 min. were required to raise the temperature to 100° and not more than 1 min. to cool to room temperature. These relatively short times could not significantly affect the results. Table 3 gives the volumes of CO_2 produced in a subsequent 5-day period at 22° when cultures of strain DL after growth at 22° for 2 days in skim milk and in broth were heat-treated as indicated in the table. These figures clearly indicate that the enzyme(s) concerned in CO_2 production was remarkably heat-stable in milk culture. It retained 100 % of its activity after heating at 80° for 60 min. and 45 % at 100° for 60 min., whereas in broth the figures were *c.* 75 and 13 % respectively.

Table 3. *Carbon dioxide produced by* 100 *ml. cultures of* Strep. diacetilactis *during* 5 *days following the killing of the* 2-*day cultures by heat treatment*

Heated at 80° for (min.) ...	5	15	30	40	60	120
	CO_2 produced (ml. N.T.P.)					
Milk culture	23	22	23	23	22	20
Broth culture	25	22	21	20	19	18
Heated at 100°						
Milk culture	22	18	13	12	11	3
Broth culture	18	15	4	3	3	2

Controls (viable organisms) in milk and in broth produced *c.* 23 and 26 ml. respectively during the 5-day period.

The possibility that prolonged heating might cause a significant chemical decarboxylation of the substrate in the medium was excluded by control experiments in which cultures were heated in the bath as described, and the gases evolved during heating were drawn off and bubbled through baryta solution. Estimation of the CO_2 evolved gave a result never exceeding 2 ml. It is a common practice in work on decarboxylation by micro-organisms to deduct from the amount of CO_2 produced by living cells the amount produced by boiled cell controls on the ground that the latter represents CO_2 due to spontaneous decarboxylation in the presence of boiled cell material. Depending on the precise heat treatment given, on the culture medium used and on the micro-organism under investigation this practice could result in appreciable error, since the results detailed above show that in some cases CO_2 production from boiled cells may arise from heat-stable enzyme systems.

Production of carbon dioxide by killed coliform bacteria

A few experiments on the same general lines as those described above were carried out with coliform bacteria in place of lactic streptococci. Cultures of all four strains after destruction by heat or disinfectants continued to produce

CO_2 as shown by the typical results for strain 13 given in Table 4. The preliminary growth period in sterilized milk was 3 days at 37° and the figures indicate that after the various treatments, which all sufficed to kill the organisms, CO_2 production at 37° in the subsequent 7 days was almost equal to that in the control. There was an indication that the decarboxylases present in coliform organisms were not quite as resistant to the treatments applied as were the decarboxylases of the lactic streptococci.

Table 4. *Carbon dioxide produced by* 100 *ml. milk cultures of a coliform organism* (*strain* 13) *during the* 7 *days following the killing of the* 3-*day cultures by various treatments.*

Method of killing	CO_2 produced (ml. N.T.P.)
Heated at 80°, 5 min.	39
Heated at 100°, 1 min.	30
$HgCl_2$, 0·2 % (w/v)	34
Cetrimide, 1·0 % (w/v)	34
Control (viable organisms)	40

DISCUSSION

It has long been known that the velocity of enzyme-controlled reactions increases with rise in temperature to a maximum at about 50° and decreases thereafter as the enzyme is progressively denatured, until at about 80° most enzymes are irreversibly denatured. Bodansky (1924) believed that in some cases heat inactivation is reversible, but few investigations into the occurrence of such heat-stable enzymes appear to have been made. The present work indicates that among bacteria, heat-resistant enzyme systems may be quite widespread. From the fact that the activity of the decarboxylative enzymes of the lactic streptococci and coliform organisms ceased above 80° but recommenced on a return to low temperature it appears that the phenomenon is due to a true reversible inactivation of the type reported by Bodansky. A few experiments indicated that the lactic streptococci of *Strep. diacetilactis* type continued to form diacetyl as well as CO_2 after killing of the organisms.

This suggests that production of other relatively complex metabolic products by heat-stable enzyme systems in killed bacteria is likely.

The stability of the decarboxylase enzyme system towards a potent protein denaturant ($HgCl_2$) and a surface-active bactericide (cetrimide) is also remarkable, and there seems to be no other recorded instance of the phenomenon.

The present findings may have a wide significance especially in commercial processes where pasteurization procedures are used to destroy bacteria. In cheese manufacture, for instance, it is generally assumed that heat treatment of milk at 72° for 15 sec. not only kills over 90 % of the bacteria present as contaminants but that it also eliminates any further influence of these organisms on the final product. Since it appears that some of the bacterial enzyme systems can survive milk pasteurization this assumption is false. The heat-stable enzyme systems may have a significant influence on the cheese-ripening process. (The production of gas, for instance, has an important bearing on the texture of cheese.) If this proves to be so the hygienic quality of the raw milk used after pasteurization for cheese manufacture is much more important than has generally been supposed because all enzymic reactions due to bacteria are not checked by the killing of the bacteria themselves.

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REFERENCES

Bodansky, A. (1924). Study of a milk-coagulating enzyme of *Solanum elaeagnifolium*. *J. biol. Chem.* **61**, 365.

Cox, G. A. & Wiley, W. J. (1939). The colorimetric estimation of diacetyl and acetoin in dairy products. *J. Coun. sci. industr. Res. Aust.* **12**, 227.

Matuzewski, T., Pijanowski, E. & Supińska, J. (1937). A new lactic streptococcus as a constituent of butter starters. *Int. Dairy Congr. Berlin*, **2**, 80.

Mellanby, J. & Woolley, V. J. (1913). The ferments of the pancreas. III. The properties of trypsin, trypsinogen and enterokinase. *J. Physiol.* **47**, 339.

Miyake, K. & Ito, M. (1924). Lethal temperature of pure Koji-diastase in aqueous solutions and recovery of its action after heating. *J. Biochem., Tokyo*, **3**, 177. (*Chem. Abstr.* 1924, **18**, 1676.)

Northrop, J. H. (1932). Reversibility of the inactivation and denaturation of trypsin by heat. *J. gen. Physiol.* **16**, 323.

Pien, J., Baisse, J. & Martin, R. (1936). Contribution à l'étude du diacetyle. *Lait*, **16**, 119.

Stapp, C. (1924). Contributions to the knowledge of bacterial enzymes. *Zbl. Bakt.* (I. Abt. Orig.), **92**, 161.

Thiel, C. C. (1940). The influence of various factors on the fermentation end-products of the hetero-fermentative Streptococci. *J. Dairy Res.* **11**, 51.

Van Slyke, D. D. & Cullen, G. E. (1914). The mode of action of urease. *J. biol. Chem.* **19**, 141.

Wilson, G. S. (1935). The bacteriological grading of milk. *Spec. Rep. Ser. med. Res. Coun. et al., Lond.* no. 206.

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Furness, G. (1952). *J. gen. Microbiol.* **7**, 335–339.

Some Factors affecting the Destruction of *Bacterium coli* by Shaking with Glass Beads

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SUMMARY: *Bacterium coli* was killed rapidly when shaken with glass beads. The bacteria were killed before they were disintegrated. The effect on this process of size of bead, speed of shaking, density of suspension, and size of container were investigated and optimal conditions for bacterial destruction were ascertained.

Curran & Evans (1942) destroyed various bacterial spores and *Bacterium coli* cells by shaking in suspension with small inert particles. They studied the effect of different kinds of particles, the proportion of these to spores and density of the spore suspension. They noted in one experiment that efficiency increased with speed of shaking.

King & Alexander (1948) amplified these observations, using glass beads, and a wider range of sporing and non-sporing bacteria. They confirmed that the optimal effect was obtained when the weight of beads in grams was the same as the volume of suspension in millilitres and that the rate of bacterial destruction increased with greater speed of shaking. They were, however, unable to sterilize a suspension of *Bact. coli*, as reported by Curran & Evans, but found that there was a proportion of all bacteria tested which was resistant to destruction by shaking with glass beads. The proportion with their strain of *Bact. coli* was 1 in 100,000. They expressed the rate of destruction as 'decimal reduction time' (D.R.T.)—time required to reduce the viable count to 1/10 of the original.

The investigations reported here were made to assess the relative importance of speed of shaking, size of bead, size of container and density of suspension in reducing the viable count and in disintegrating the bacteria, and to determine the most effective combination of these factors.

METHODS

The shaker had adjustable set speeds between 430 and 700 double horizontal excursions or shakes/minute (s.p.m.) at an amplitude of 31 mm. Four bottles could be shaken at once which facilitated comparative tests. Airflow over the containers prevented undue rise in temperature.

Glass beads (Messrs Chance Bros. Ltd., Smethwick)—Ballotini grades 9, 12 and 16—were used. They had mean diameters of 337, 171 and 107 μ., respectively, based on the measurement of 100 beads. The distribution of sizes in each grade is shown in Table 1. Before use they were soaked in chromic-sulphuric acid mixture, thoroughly washed in water, dried and sterilized in the hot-air oven. Twenty-five g. were added to 25 ml. of suspension.

Table 1. *The distribution of diameters of glass beads within each grade*

Grade 9		Grade 12		Grade 16	
Diameter (μ.)	%	Diameter (μ.)	%	Diameter (μ.)	%
<260	3	<100	5	<80	8
261–280	10	101–120	9	81–90	6
281–300	13	121–140	15	91–100	17
301–320	18	141–160	16	101–110	24
321–340	11	161–180	15	111–120	23
341–360	12	181–200	16	121–130	14
361–380	14	201–220	13	131–140	8
381–400	15	221–240	9	>140	0
>400	4	>240	2	—	—

Three sizes of bottle were used: (*a*) a screw-capped '10 oz. flat' with the neck cut off about 12 mm. from the shoulder, holding 300 ml. It was placed on its narrow side; the internal distance between the sides, in the line of excursion, was about 30 mm.; (*b*) a bottle with square base, internal distance between sides about 30 mm., 11·2 cm. to the shoulder, holding 130 ml. It was placed upright; (*c*) a square-based bottle, internal distance between sides about 50 mm., 13 cm. to the shoulder, holding 420 ml. placed on its side, transversely to the line of excursion. The bottles were stoppered with pressure tubing dilated to the correct size with a glass rod and inserted to the level of the shoulder of the bottle. There was always some suspension in a thin layer around the stopper which, when the stopper was removed after shaking, mixed with the contents of the bottle, and by adding living bacteria, introduced an error in estimating the decrease of viable counts caused by shaking, especially when the contents had been nearly sterilized.

Suspensions of *Bact. coli* were made in distilled water from agar plate cultures incubated at 37° for 18 hr.; they were equal in opacity to Brown's no. 1 tube (Burroughs, Wellcome and Co.) and contained about 800×10^6 organisms/ml. To break up clumps of cells, 100 ml. of suspension was shaken in bottle (*a*) at 600 s.p.m. for 10 min. This appeared to be the optimal time; shaking for a further 15 min. decreased the viable count by 5–6 %. A similar suspension kept at room temperature for 4 hr. showed an increase in the viable count from 620 to 870×10^6. The conditions of suspension were therefore not deleterious.

Viable counts were made in duplicate by placing 1 ml. of suitable dilution in a Petri dish, mixing with 15 ml. agar, melted and cooled to 50°, incubating for 24 hr. at 37° and counting with a × 2½ lens. Incubation for 48 hr. did not increase the count.

After breaking up the clumps, 25 ml. of suspension was transferred to a bottle for experimental shaking. The logarithm of the number of organisms surviving after several periods of shaking was plotted and from the graph the D.R.T. ascertained. This procedure facilitated the comparison of results in successive experiments with suspensions containing different numbers of organisms.

Factors affecting the rate of killing of Bacterium coli

Shaking alone. Shaking without beads for 3 hr. at 530, 600 and 700 s.p.m. decreased the viable count by 16·5, 20·7 and 19·5 % respectively. Similar results were obtained with each size of bottle. This diminution was small compared with shaking with beads.

Size of bead. The addition of beads of any of the sizes tested shortened the D.R.T. markedly as compared with shaking alone. With each speed of shaking, decreasing the size of the beads shortened the D.R.T. (Table 2). When the logarithm of the number of surviving organisms was plotted against the time shaken, the decrease followed the exponential law until the viable count reached a figure between log 2 and log 3, after which there was a decline in the rate of destruction. On plotting the D.R.T. against mean diameter of bead for each speed separately, the points fell in each case on a straight line, indicating a linear relationship. Thus for any beads having a mean diameter within the range 107–337 μ. the rate of destruction can be calculated. Beads with a still smaller mean diameter were not obtainable, but it seems probable that further decrease in size would have reached a limit of increasing effectiveness.

Table 2. *The effect of size of bead and speed of shaking on the killing of* Bacterium coli

Shakes per min.	Bead grade 9 Type of bottle			Bead grade 12 Type of bottle			Bead grade 16 Type of bottle		
	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)	(*a*)	(*b*)	(*c*)
	D.R.T. (time in min. to reduce count by 90 %)								
530	46	47	47	21·5	21	23	13	13	12
600	27	27	27	13	12	13	6	6·5	6
700	26	25	27	8·5	9	9	3·5	3·5	3·5

Density of cell suspension. Suspensions of different densities were shaken simultaneously at 600 s.p.m. with grade sixteen beads. The D.R.T. of suspensions up to 1335×10^6 cells/ml. was 6 min. Increasing this density tenfold prolonged the D.R.T. to 10 min.

Speed of shaking. Increase in speed between 530 and 700 s.p.m. shortened the D.R.T. for two of the three grades of beads tested (Table 3); grade 9 exerted its maximum efficiency at 600 s.p.m., the slight difference at 700 s.p.m. being within experimental error.

Size of container. The amount of suspension was always 25 ml. which, stated as a proportion of the volume of bottles (*a*), (*b*) and (*c*) respectively, was 1 : 12, 1 : 5·2 and 1 : 16·8. The distance between the sides of the bottles (*a*) and (*b*), in the direction of the movement, was the same as the amplitude of the excursion, for bottle (*c*) it was nearly twice as great. The results (Table 2) indicate, however, that the differences in the three bottles used had no significant effect on the result as similar results were obtained with all bottles for each size of bead.

A comparison of total and viable counts

Comparisons were made of total counts, using a counting chamber, and viable counts using pour plates prepared from the same dilutions. In every case the total count was higher than the viable count, indicating that the beads first killed the organisms without apparent injury as viewed in stained films, by dark ground illumination, and in hanging-drop preparations; disintegration followed later. Differences between the two counts depended on the size of the bead. The rate of destruction followed the exponential law. With grade 9 beads the ratio of total to viable counts averaged 6 : 1 or 8 : 1 at 600 s.p.m.; with the smaller beads the ratio was greater. With decreasing sizes of beads the killing increased faster than the disintegrating effect.

Effect of prolonged shaking with beads

Attempts were made to sterilize suspensions of *Bact. coli* by prolonged shaking (Table 3). Complete sterilization was not obtained, but the numbers of viable organisms that remained were very small. In Exp. 3 a suspension containing 63,200 × 10^6 organisms/ml. after 3 hr. shaking with grade 12 beads at 600 s.p.m. contained only 3–4 organisms/ml. In Exps. 4 and 6 the bottle was stoppered with pressure tubing which had been dilated to the correct size with glass tubing and the whole covered with a rubber membrane. The bottle was sampled once after shaking. A hypodermic needle was inserted through the glass tube, the membrane pierced, and several ml. of the suspension withdrawn. Although this method avoided removing the bung and consequent liberation of bacteria which had been protected from the beads in films and crevices around the bung, there was the possibility that the few remaining viable bacteria had escaped destruction in such situations; alternatively they might represent the last few bacteria which only very prolonged shaking would eradicate.

Table 3. *The destruction of* Bacterium coli *by prolonged shaking with beads*

Exp. no.	Shakes per min.	Bead grade	Time shaken (hr.)	Viable count (organisms/ml.) Before shaking	Viable count (organisms/ml.) After shaking
1	530	12	3	15 × 10^6	125
2	530	16	3	1070 × 10^6	24
3	600	12	3	63,200 × 10^6	3·5
4	600	16	4	450 × 10^6	2·5
5	600	16	1½	630 × 10^6	9·5
6	600	16	1½	545 × 10^6	10·5

DISCUSSION

Although the results were not affected by variations in size of container and by the proportion of the volume occupied by suspension, the bottles were never more than one fifth full and the distance between the vertical sides was never less than the excursion of the shake or greater than twice the distance. It is therefore possible that nearly full bottles and bottles outside these limits might influence the results.

The limit of effectiveness of speed of shaking was reached only with the larger beads, grade 9. There would no doubt be a limit to the increasing efficiency of higher speeds of shaking and of smaller beads but for technical reasons these have not been investigated. With our strain of *Bact. coli*, however, the number of viable organisms was decreased to as few as 2·5 organisms/ml. after 4 hr. shaking and there does not appear to be a percentage of resistant cells as reported by King & Alexander (1948).

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REFERENCES

Curran, H. R. & Evans, F. R. (1942). The killing of bacterial spores in fluids by agitation with small inert particles. *J. Bact.* **43**, 125.

King, H. K. & Alexander, H. (1948). The mechanical destruction of bacteria. *J. gen. Microbiol.* **2**, 315.

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Kleczkowski, J. & Kleczkowski, A. (1952). *J. gen. Microbiol.* 7, 340–349.

Effect of Specific Polysaccharides from the Host Bacteria and of Ribonuclease on the Multiplication of Rhizobium Phages

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SUMMARY: Two serologically unrelated strains of nodule bacteria produced two different polysaccharides, only one of which precipitated with antiserum to its parent bacterium. Both polysaccharides interfered with the multiplication of two bacteriophages in liquid cultures of the two bacterial strains, each of which was susceptible to only one of the two bacteriophages. One polysaccharide was slightly more effective than the other in interfering with multiplication of both bacteriophages; one phage was much more susceptible than the other to the interfering action of both polysaccharides. Crystallized pancreatic ribonuclease interfered with multiplication of bacteriophages much more strongly than did the polysaccharides. Neither the polysaccharides nor ribonuclease destroyed the phage particles.

The chance that a bacteriophage particle will multiply in a liquid culture of Rhizobium bacteria decreases with increasing age of the culture (Kleczkowski & Kleczkowski, 1951). This can have various causes. Bacteria may become increasingly resistant to infection as their cultures age, or some metabolic products accumulating in the medium might interfere with phage activity. The literature contains many references suggesting that polysaccharides, or materials containing polysaccharides, produced by individual bacterial species specifically interfere with the activity of bacteriophages that attack those species (Levine & Frisch, 1933; Burnet, 1934; Gough & Burnet, 1934; White, 1936; Miller & Goebel, 1949). Some authors have also related polysaccharides capable of interfering specifically with bacteriophages to those that confer serological specificity to their host bacteria (see Burnet, Keogh & Lush, 1937). On the other hand, Ashenburg, Sandholzer, Scherp & Berry (1940) found no evidence for specific inhibition of activity of bacteriophage by a polysaccharide produced by its host bacteria, for polysaccharides of widely different origins inhibited similarly. Some polysaccharides have been found to interfere with the multiplication of certain animal viruses in the allantoic sac of the chick embryo (Green & Wooley, 1947; Ginsberg, Goebel & Horsfall, 1948) and with infectivity of plant viruses (Takahashi, 1942, 1946; Bawden & Freeman, 1952). There is no evidence of any specificity in the action of polysaccharides against individual viruses, but different plants differ in the degree to which their susceptibility is influenced (Bawden & Freeman, 1952).

The work described in the present paper was done to see whether strains of Rhizobium bacteria that differ serologically and in their susceptibility to different bacteriophages, produce different specific polysaccharides and, if so, whether these are active serologically and act specifically against a bacteriophage to which the species is susceptible.

For comparison, tests were made to see whether pancreatic ribonuclease, which is known to be a powerful inhibitor of activity of plant viruses (Loring, 1942; Kassanis & Kleczkowski, 1948), has any effect on activity of Rhizobium bacteriophages. It reduces the numbers of lesions formed by plant viruses on leaves of susceptible plants, but has no effect on numbers of plaques formed by one of the phages (Kassanis & Kleczkowski, 1948).

MATERIAL AND METHODS

Two strains of nodule bacteria, 317 derived from pea (obtained from the Agricultural Research Station, Wisconsin) and Cl_5 from clover and two homologous bacteriophages were used. Each bacterial strain was susceptible only to its homologous phage and was agglutinated only by its homologous antiserum. The phages were originally obtained from soil and isolated from single plaques. The media, the poured plate method of obtaining plaques, and other methods of cultivation were the same as used previously (Kleczkowski & Kleczkowski, 1951).

The polysaccharides were isolated from 3-week-old, liquid cultures of the bacteria. The cultures were centrifuged, 10 ml. 20 % NaCl were added to each litre of the clear supernatant fluid followed by 2 l. 96 % (v/v) ethanol. The precipitate was filtered off and suspended in 100 ml. of a 20 % solution of sodium acetate neutralized with glacial acetic acid. Material that did not dissolve within 24 hr. was removed by centrifugation. Further procedure was the same as that described by Heidelberger, Kendall & Scherp (1936) for isolating pneumococcal polysaccharides, except that, before removing protein by Sevag's procedure, the material was precipitated with ethanol, dissolved in 0·06 M-phosphate buffer at pH 7·0 and incubated for 24 hr. at 37° with the addition of $\frac{1}{30}$ vol. of filtered saliva. (This removed a material which gave the same colour as glycogen on treatment with iodine.) The polysaccharide was then precipitated with ethanol, dissolved in acidified 20 % solution of sodium acetate, and Sevag's treatment was applied. (This removed a material that gave a positive biuret test). The polysaccharide was precipitated from the resulting solution by ethanol, washed in 96 % ethanol, in absolute ethanol, in ether, dried and ground to a white powder. The yield was about 100 mg. from 1 l. bacterial culture.

Antisera were produced by injecting rabbits intraveneously with bacterial suspensions prepared by washing with physiological saline 3- to 5-day old cultures from agar slopes. Agglutinin tests with bacterial suspensions were made as previously described (Kleczkowski & Thornton, 1944). Precipitin tests with the bacterial polysaccharides were made similarly to the agglutinin tests except that the antiserum was used at a constant dilution and the polysaccharide concentration varied.

Crystallized beef pancreatic ribonuclease was prepared by the method described by Kunitz (1940).

For paper chromatography the polysaccharides were hydrolysed from 20 min. to $2\frac{1}{2}$ hr. at 120° in the presence of 5 % H_2SO_4, which was then removed by adding an excess of $BaCO_3$, and centrifuged after a few hr. incubation at room temperature.

One-dimensional chromatograms were prepared as described by Partridge (1948), using two different solvents; water-saturated phenol (with ammonium-saturated atmosphere) and a water-saturated *n*-butanol-ethanol mixture with the volume ratio butanol : ethanol : water = 4·5 : 0·5 : 5·0.

Benzidine reagent (Horrocks, 1949) was used as a developer. For the quantitative estimation of sugars from the different spots after development, water extracts were made from areas corresponding with the spots but cut from part of the filter-paper that had not been sprayed with the developer. The reducing power of the extract was then determined by the Somogyi (1945) titrimetric procedure.

RESULTS

Some properties of the polysaccharides

Both polysaccharides were obtained as white powders, which formed clear colourless solutions in water and were highly viscous at 1 %. Solutions of polysaccharide 317 were obviously more viscous than those of Cl_5. They contained no nitrogen, did not stain with iodine and did not reduce Fehling's solution unless previously hydrolysed. After hydrolysis with 5 % H_2SO_4 for 1 hr. at 120° and subsequent neutralization with NaOH, their reducing power was about 85 % that of glucose. The intensity of colour developed when solutions of polysaccharides were heated with ten times their volume of 0·2 % orcinol in 66 % H_2SO_4, did not differ appreciably from that formed under the same conditions by an equally concentrated solution of glucose.

The polysaccharides had different compositions. The 317 produced only two spots on the paper chromatograms. Their positions corresponded with those of glucose and of a uronic acid. The Cl_5 produced three spots; the positions of two of them were identical with those of the components of the 317, that of the third corresponded with the position of fructose. This spot was weak, and it was absent if the time of hydrolysis was 2½ hr., and the temperature was raised to 125°, under which conditions fructose is destroyed and gives no spot on the chromatogram. Over 90 % of the total reducing power of each hydrolysed polysaccharide was due to the component identified as glucose.

If hydrolysed for only 20 min., the hydrolysate of each polysaccharide contained a component that gave an additional sport on chromatograms. This occurred only when phenol was used as a solvent, and then the component moved very slowly (R_F about 0·015). The component was presumably stationary with the butanol-ethanol mixture so that its position coincided with that of the uronic acid. The component may be an aldobionic acid or an oligosaccharide that was hydrolysed when hydrolysis was prolonged.

Our results agree with those obtained by previous workers who examined polysaccharides produced by strains of nodule bacteria and found they contained glucose and glucuronic acid, the latter probably forming part of the aldobionic acid (Hopkins, Peterson & Fred, 1931; Cooper, Daker & Stacey, 1938; Schlüchterer & Stacey, 1945).

The two polysaccharides differed serologically. The 317 polysaccharide precipitated with an antiserum to 317 bacteria (Table 1) but not with an antiserum to Cl_5 bacteria. The polysaccharide Cl_5 did not precipitate with either antiserum. Each antiserum agglutinated suspensions of its homologous bacterium up to a titre of 1/6000, but neither of the bacterial strains was agglutinated by antiserum to the other.

Although polysaccharides produced by different strains of clover *Rhizobium* may differ serologically, some of them may be closely related to polysaccharides produced by bacteria that do not belong to the Rhizobium group. The polysaccharide isolated by Schlüchterer & Stacey (1945) from 'Bartel A' strain of clover *Rhizobium* was found by Dr M. Heidelberger to give a positive precipitin test at a high dilution with pneumococcus III antiserum and also with mixed antisera to other types of pneumococcus.

The possibility that one or both of the polysaccharides 317 and Cl_5 was a mixture cannot be excluded. However, the high dilution end point at which the polysaccharide 317 precipitated with the antiserum to 317 bacteria makes it unlikely that the polysaccharide was a mixture of which the serologically active component was only a minor part.

Effect of the polysaccharides and of ribonuclease on the bacteriophages

Three types of experiment were made to see whether the polysaccharides and ribonuclease interfere with bacteriophage activity. Experiments of the first type were designed to show whether contact with these materials permanently inactivates phage particles. Bacteriophage 317 was incubated with the polysaccharide 317 and with ribonuclease, then diluted to obtain a conveniently countable number of plaques, mixed with host bacteria and plated. The details and results are given in Tables 2 and 3 which show that the incubation did not inactivate any phage particles.

Experiments of the second type were designed to test whether the presence of the polysaccharide or ribonuclease in the bacterial culture, to which a suitably diluted phage preparation was added and which was then plated directly, could render some phage particles inactive. Table 4 gives the details of one such experiment and shows that the polysaccharides had no such effect, for the number of plaques formed was not affected.

Experiments of the third type were designed to show whether the presence of the polysaccharide or of ribonuclease in a liquid culture of host bacteria interferes with phage multiplication in the culture. Table 5 shows that the polysaccharide 317, added to a concentration of 0·25% to a culture of 317 bacteria, which was then inoculated with 317 bacteriophage, did interfere with multiplication of the phage, which, within the first 3 hr., increased only about 50 times compared to about 100 times in the control. The polysaccharide Cl_5 interfered with multiplication of 317 phage only slightly, though significantly, less than did the polysaccharide 317. Potato starch also interfered

Table 1. *Precipitin reaction of the polysaccharide 317 with an antiserum to 317 bacteria*

Time of reading (hr.)	Reciprocal of dilution of a 0·05 % solution of the polysaccharide										
	1	2	4	8	16	32	64	128	256	512	1024
1	–	–	±	++	+	+	±	–	–	–	–
2	–	–	+	+++	+++	++	++	+	±	–	–
4	–	+	+++	+++	+++	+++	++	++	+	±	–

1 ml. of the antiserum diluted 1/10 was added to 1 ml. of variously diluted solution of the polysaccharide, and incubated at 50°. +++, ++, + and ± signs show the presence and the extent of precipitation; – sign shows the absence of precipitation.

Table 2. *Incubation of bacteriophage 317 with the polysaccharide 317 before mixing with host bacteria and plating*

	Composition of the mixtures			
Mixture no.	0·5 % solution polysaccharide (ml.)	H_2O (ml.)	Phage culture diluted 1/50 (ml.)	Mean no. plaques/plate
1	1·0	—	1·0	41
2	—	1·0	1·0	38

The mixtures were incubated for 1 hr. at room temperature, then diluted 10^{-5} in 24 hr. bacterial cultures and plated. Four platings were made with each mixture.

Table 3. *Incubation of bacteriophage 317 with ribonuclease before mixing with host bacteria and plating*

	Composition of the mixtures			Mean no. plaques/plate formed by mixture incubated for	
Mixture no.	0·4 % ribonuclease (ml.)	H_2O (ml.)	Phage culture dil. 10^{-4} (ml.)	0 hr.	24 hr.
1	0·25	0·75	0·1	103	95
2	—	1·00	0·1	106	104

The mixtures were incubated for 24 hr. at 25°. Samples were taken at the beginning and at the end of incubation, diluted 1/100 in 24 hr. bacterial cultures and plated. Each plating was made in four replications.

although considerably less. Rabbit serum albumin used at the same concentration had no effect.

Ribonuclease when present at a concentration of 0·01 %, i.e. at $\frac{1}{25}$th of the concentration of the polysaccharides, inhibited phage multiplication much more strongly than did the polysaccharides, and at a concentration of 0·1 % it stopped its multiplication completely during the first 3 hr.

After 24 hr. incubation the ratios of the concentrations reached by bacteriophage in the presence of the interfering materials to that reached in their absence were the same as, or greater than, after 3 hr. incubation. It is obvious, therefore, that the inhibiting effect of the materials tested lasted less than 24 hr.

Preparations of crystallized ribonuclease obtained from beef pancreas by Kunitz's (1940) method contain a proteolytic enzyme (Kleczkowski, 1948) and possibly some other enzymes and proteins. Proteolytic activity of the preparations can be destroyed or greatly decreased by heating for 5 min. to 90–100° at pH 7·5, preserving about 30 % of the original ribonuclease activity (Kleczkowski, 1948). As almost all known enzymes are also destroyed by heating in these conditions, heated preparations of crystallized ribonuclease can be assumed to be free from active enzymes other than ribonuclease. After heating in 0·06 M-phosphate buffer at pH 7·5 for 5 min. in a water-bath at 100°, the ribonuclease preparation still interfered with phage multiplication to an extent proportional to its remaining ribonuclease activity.

The results given in Table 6 show that the extent of inhibition depends on the host bacterium. The growth of phage Cl_5 in Cl_5 bacterial culture was much more strongly inhibited by polysaccharides 317 and Cl_5 than was the growth of phage 317 in a culture of 317 bacteria. Again the polysaccharide 317 inhibited somewhat more strongly than did the polysaccharide Cl_5.

To see whether inhibition of phage multiplication by the tested materials could be a direct result of inhibition of growth of host bacteria, 24 hr. bacterial cultures were incubated at 25° with the materials at concentrations at which they inhibited phage growth. Samples were taken at intervals for haemocytometer counts. The presence of 0·25 % of the polysaccharides or of 0·01 % ribonuclease had no effect on multiplication of the bacteria, but the presence of 0·1 % ribonuclease decreased it considerably.

Experiments were also made to test whether the polysaccharide 317 interferes with infectivity of tobacco mosaic virus. Details and results of one of such experiments are given in Table 7, which shows that the presence of the polysaccharide in the inoculum reduced numbers of lesions formed on leaves of *Nicotiana glutinosa* more than did the presence of potato starch or rabbit serum albumin. The reduction was very slight by comparison with that caused by the polysaccharide obtained from yeast by Takahashi (1942), or by such substances as beef pancreatic ribonuclease or a protein isolated from *Phytolacca esculenta* (Kassanis & Kleczkowski, 1948).

Table 4. *Effect of adding polysaccharide* 317 *or ribonuclease to bacterial cultures with which bacteriophage* 317 *was plated*

Mixture no.	Composition of the mixtures: 1 % solution polysaccharide (ml.)	0·4 % solution ribonuclease (ml.)	H_2O (ml.)	24 hr. bacterial culture (ml.)	Phage culture diluted 10^{-6} (ml.)	Mean no. plaques/plate
1	0·25	—	—	0·75	0·1	38
2	—	0·25	—	0·75	0·1	31
3	—	—	0·25	0·75	0·1	33

The mixtures were mixed with 10 ml. molten agar medium, which had been cooled to 42°, and poured into Petri dishes. Four plates were made from each mixture.

Table 5. *Effect of the polysaccharides and of ribonuclease on the multiplication of* 317 *bacteriophage*

Composition of the mixtures	Concentration of bacteriophage in terms of no. of plaques/ml. of the mixtures: Immediately	After 3 hr.	After 24 hr.
3 ml. 24 hr. culture of 317 bacteria + 1 ml. of:			
1 % 317 polysaccharide	21×10^2	10×10^4	65×10^8
1 % Cl_5 polysaccharide	18×10^2	13×10^4	85×10^8
1 % potato starch	20×10^2	17×10^4	106×10^8
1 % rabbit serum albumin	19×10^2	24×10^4	120×10^8
0·4 % ribonuclease	17×10^2	15×10^2	$4·5 \times 10^8$
0·04 % ribonuclease	17×10^2	26×10^2	46×10^8
0·004 % ribonuclease	19×10^2	23×10^4	125×10^8
H_2O	19×10^2	25×10^4	129×10^8

0·4 ml. of a culture of phage 317 diluted 10^{-5} was added to each mixture. The mixtures were then incubated at 25° and samples were taken at intervals for phage estimation.

Table 6. *Effect of polysaccharides* (*PSH*) *on multiplication of* Cl_5 *bacteriophage*

Mixture no.	Composition of mixtures: 24 hr. culture Cl_5 bacteria (ml.)	1 % 317 PSH (ml.)	1 % Cl_5 PSH (ml.)	H_2O (ml.)	Phage Cl_5 diluted 10^{-3} (ml.)	Concentration of bacteriophage in terms of no. plaques/ml. of the mixtures: Immediately	After 24 hr.
1	0·75	0·25	—	—	0·1	71×10^2	29×10^5
2	0·75	—	0·25	—	0·1	76×10^2	67×10^5
3	0·75	—	—	0·25	0·1	67×10^2	1050×10^5

The mixtures were incubated at 25° and samples were taken at stated intervals for phage estimation.

Table 7. *Effect of polysaccharide* 317 *on activity of tobacco mosaic virus*

Addition to the inoculum	Mean no. lesions/half-leaf with virus at 5 mg./l.	Mean no. lesions/half-leaf with virus at 0·5 mg./l.
0	93	8·5
0·1 % 317 polysaccharide	23	2
0·05 % 317 polysaccharide	49	5·5
0·025 % 317 polysaccharide	73	7·5
0·1 % potato starch	72	7
0·05 % potato starch	76	—
0·1 % rabbit serum albumin	—	4

Each inoculum was applied to twelve half-leaves distributed among six *Nicotiana glutinosa* plants according to the principle of the Latin square. Each column of numbers was obtained in a separate experiment.

DISCUSSION

The fact that specific bacterial polysaccharides can interfere with phage multiplication shows clearly that their accumulation in a liquid bacterial culture may decrease the chance of a phage particle to multiply. However, the ability to interfere with phage multiplication is shared by various other materials such as potato starch and beef pancreatic ribonuclease, the last one being by far the most effective. This suggests that, apart from specific polysaccharides, various other products of bacterial metabolism that accumulate in the medium are also likely to decrease the chance of a phage particle to multiply.

There is obviously no specific affinity between the bacterial polysaccharides and phage particles, because interference by the polysaccharides with phage multiplication is unspecific, i.e. not restricted to phages whose hosts produced the polysaccharide. Also it is not connected with the ability of the polysaccharides to precipitate specifically with antisera to host bacteria. The unspecific nature of the interference is further emphasized by the fact that the polysaccharides can also interfere with infectivity of tobacco mosaic virus.

The mechanism of interference by the polysaccharides or ribonuclease remains unknown, but some possibilities can be excluded. First, phage particles are not destroyed by these interfering materials. Secondly, the materials do not interfere with phage multiplication by affecting the rate of bacterial multiplication. It is true that ribonuclease, when present at a concentration of 0·1 % did interfere with bacterial multiplication, but it did not do so at 0·01 %, although it interfered strongly with phage multiplication at this concentration. It is possible, therefore, that ribonuclease interferes with phage multiplication by affecting bacterial metabolism. The latter is obviously affected by 0·1 % ribonuclease. It may therefore also be affected by 0·01 % ribonuclease though not sufficiently to influence bacterial multiplication and it may be similarly affected by the polysaccharides. It is also possible, however, that 0·01 % ribonuclease affects only the bacterial surface, making it less penetrable for bacteriophage, or preventing its adsorption; and the polysaccharides may interfere with phage activity by forming a protective coating on the bacterial surface.

A feature of the inhibitory effect of the polysaccharides and of ribonuclease on phage multiplication is that with young bacterial cultures it is only temporary; with 24 hr. cultures of 317 bacteria inoculated with 317 phage it lasts less than 24 hr. Various explanations can be suggested. For example, if the inhibitory mechanism is based on a protective coating of the bacterial surface, in a young fast-growing culture the surface may outgrow its protective coating and thus increase its vulnerability; if it is based on interference with bacterial metabolism, the metabolism may recover its original course either by adaptation or through some process directed against the inhibitor.

Although the interference by the polysaccharides is unspecific, different host-phage systems are affected to different extents. This is shown by the fact that the phage Cl_5 was much more affected by each of the two polysaccharides, 317 and Cl_5, than was the phage 317.

It may seem that there is a contradiction between a polysaccharide or ribonuclease interfering with phage multiplication in a liquid bacterial culture and having no effect on the numbers of plaques formed on a solid medium. The contradiction, however, is apparent only. To be plated, a phage-bacteria mixture is diluted 1/10 in molten agar medium, and this may suffice to make the inhibitor ineffective. However, should it remain effective, plaque numbers are still unlikely to be affected, because the inhibitor does not destroy phage particles or have more than a temporary effect on phage multiplication in a young bacterial culture.

Bawden & Freeman (1952) have discussed the interference by a polysaccharide and by ribonuclease with the activity of some plant viruses, and have concluded that it is most likely to occur because these substances affect the metabolism of the host cells. Thus there may be no basic difference between the effect of polysaccharides and of ribonuclease on the activity of plant viruses, on the one hand, and on that of bacteriophage, on the other. If so, the fact that these materials decrease the numbers of local lesions formed by plant viruses and have no effect on the numbers of plaques formed by a bacteriophage would mean that the lesions are not analogous to the plaques. There would be a closer analogy between the formation of lesions and lysis of liquid bacterial cultures. This analogy is also apparent when a comparison is made between the dilution curves obtained with numbers of lesions caused by plant viruses and the dilution curves obtained with proportions of liquid bacterial cultures lysed by a bacteriophage (Kleczkowski & Kleczkowski, 1951).

REFERENCES

Ashenburg, N. J., Sandholzer, L. A., Scherp, H. W. & Berry, G. P. (1940). The influence of bacterial and non-bacterial polysaccharides upon bacteriophagy. *J. Bact.* **39**, 71.

Bawden, F. C. & Freeman, G. G. (1952). The nature and behaviour of inhibitors produced by *Trichothecium roseum* Link. *J. gen. Microbiol.* **7**, 154.

Burnet, F. M. (1934). The phage-inactivating agent of bacterial extracts. *J. Path. Bact.* **38**, 285.

Burnet, F. M., Keogh, E. V. & Lush, D. (1937). Immunological reactions of filterable viruses. *Aust. J. exp. Biol. med. Sci.* **15**, 227.

Cooper, E. A., Daker, W. D. & Stacey, M. (1938). Enzyme formation and polysaccharide synthesis by bacteria. III. Polysaccharides produced by 'nitrogen fixing' organisms. *Biochem. J.* **32**, 1752.

Ginsberg, H. S., Goebel, W. F. & Horsfall, F. L. (1948). The inhibitory effect of polysaccharide on mumps multiplication. *J. exp. Med.* **87**, 385.

Gough, G. A. C. & Burnet, F. M. (1934). The chemical nature of the phage-inactivating agent in bacterial extracts. *J. Path. Bact.* **38**, 301.

Green, R. M. & Wooley, D. W. (1947). Inhibition by certain polysaccharides of haemagglutination and multiplication of influenza virus. *J. exp. Med.* **86**, 55.

Heidelberger, M., Kendall, F. E. & Scherp, H. W. (1936). Specific polysaccharides of types I, II and III pneumococcus. *J. exp. Med.* **64**, 559.

Hopkins, E. W., Peterson, W. H. & Fred, E. B. (1931). Glucoronic acid, a constituent of the gum of root nodule bacteria. *J. Amer. chem. Soc.* **53**, 306.

Horrocks, R. H. (1949). Paper partition chromatography of reducing sugars with benzidine as a spraying reagent. *Nature, Lond.* **164**, 444.

Kassanis, B. & Kleczkowski, A. (1948). The isolation and some properties of a virus-inhibiting protein from *Phytolacca esculenta*. *J. gen. Microbiol.* **2**, 143.

Kleczkowski, A. (1948). Proteolytic activity of preparations of crystallized ribonuclease. *Biochem. J.* **42**, 523.

Kleczkowski, A. & Kleczkowski, J. (1951). The ability of single phage particles to form plaques and to multiply in liquid cultures. *J. gen. Microbiol.* **5**, 346.

Kleczkowski, A. & Thornton, H. G. (1944). A serological study of root nodule bacteria from pea and clover inoculation groups. *J. Bact.* **48**, 661.

Kunitz, M. (1940). Crystalline ribonuclease. *J. gen. Physiol.* **24**, 15.

Levine, P. & Frisch, A. W. (1933). Specific inhibition of bacteriophage by bacterial extracts. *Proc. Soc. exp. Biol., N.Y.* **30**, 993.

Loring, H. S. (1942). The reversible inactivation of tobacco mosaic virus by crystalline ribonuclease. *J. gen. Physiol.* **25**, 497.

Miller, E. M. & Goebel, W. F. (1949). Studies on bacteriophage. I. The relationship between the somatic antigens of *Shigella sonnei* and their susceptibility to bacterial viruses. *J. exp. Med.* **90**, 255.

Partridge, S. M. (1948). Filter paper partition chromatography of sugars. *Biochem. J.* **42**, 238.

Schlüchterer, E. & Stacey, M. (1945). The capsular polysaccharide of *Rhizobium radicicolum*. *J. chem. Soc.* 776.

Somogyi, M. (1945). A new reagent for the determination of sugars. *J. biol. Chem.* **160**, 61.

Takahashi, W. W. (1942). A virus inactivator from yeast. *Science*, **95**, 586.

Takahashi, W. W. (1946). Properties of a virus inactivator from yeast. *Science*, **104**, 377.

White, P. B. (1936). Differential fixation of cholera phages by extracts of *V. cholerae*. *J. Path. Bact.* **43**, 591.

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HALL, E. R. (1952). *J. gen. Microbiol.* 7, 350–357.

Investigations on the Microbiology of Cellulose Utilization in Domestic Rabbits

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SUMMARY: Cellulolytic iodophilic cocci have been isolated in significant numbers from rabbit caecal contents. Seven strains of the anaerobic Gram-positive cocci have been isolated on seven separate occasions from rabbits obtained from different sources and maintained on various diets. No other cellulolytic bacteria have been observed in cultures inoculated with high dilutions of caecal contents, suggesting that the isolated cocci are the most important agents of cellulose digestion in the caecum.

Of the carbohydrates tested the caecal cocci utilize only cellulose and cellobiose. The products of cellulose fermentation include acetic acid, succinic acid, a trace of formic acid, ethanol, CO_2 and H_2.

It is generally accepted that the primary digestion of cellulose in herbivorous mammals is accomplished by micro-organisms indigenous to the host. The demonstration by Elsden, Hitchcock, Marshall & Phillipson (1946) of volatile fatty acids in the rumen of cattle and sheep and the isolation of rumen cellulolytic bacteria in significant numbers (Hungate, 1950) substantiate this concept. A similarity between the mechanism of cellulose digestion in ruminants and rabbits is suggested by the presence of volatile fatty acids in rabbit caecal contents as shown by Elsden *et al.* (1946) and by the results of direct microscopic examination of rabbit caecal contents by Baker & Martin (1937), who reported that various morphological types of bacteria were active in the disintegration of plant cell walls in the rabbit caecum.

The present investigation was undertaken in order to provide experimental evidence concerning the cultural and physiological characteristics of the cellulolytic bacteria found in the rabbit caecum.

EXPERIMENTAL

Culture methods

The culture methods employed in the isolation of cellulose-digesting bacteria from the caecum of domestic rabbits were similar to those used by Hungate (1950) in his studies of mesophilic anaerobic cellulose-decomposing bacteria. The basic culture medium contained the following final percentages (w/v) of ingredients: NaCl, 0·025; $(NH_4)_2SO_4$, 0·01; K_2HPO_4, 0·02; KH_2PO_4, 0·01; $CaCl_2$, 0·003; $MgSO_4.7H_2O$, 0·003; $NaHCO_3$, 0·5; sodium thiolacetate, 0·02 or L-cysteine monohydrochloride, 0·01. Either cotton or filter-paper cellulose in the form of a fine suspension was added in concentration of 0·1–0·2 % (w/v) in liquid and 0·6 % (w/v) in agar media. Rumen fluid or caecal extract in a concentration of 20–30 % (v/v) was included in all culture media. Triple distilled water was used to bring the total volume of the culture medium to 100 ml. The medium minus $NaHCO_3$ was prepared and sterilized in 200 ml. Florence flasks. After sterilization, the $NaHCO_3$ solution which had been sterilized by filtration, was added and the medium transferred to sterile rubber-stoppered tubes. The final pH value of the medium was 6·8–7·1. Anaerobiosis was maintained during preparation and transfer of the medium by passing oxygen-free CO_2 or N_2 through it and the receiving tubes.

The inoculum consisted of caecal contents removed immediately after killing the rabbit with ether. The entire caecum was removed, weighed, and the contents stripped into a flask containing 50–100 ml. of sterile basal culture medium. After mixing the caecal contents with the diluent, serial dilutions were made into both liquid and solid cellulose media. The weight of caecal contents was determined by the difference between the original weight of the caecum and its weight after being stripped.

Pure cultures of the cellulolytic cocci were obtained from agar cultures of high dilution by picking a colony with a glass capillary pipette and transferring it to a tube of melted and cooled rumen fluid cellobiose agar. The colony was broken against the side of the tube and mixed with the agar, serial dilutions were then made into four or five tubes of the same medium. When discrete colonies were apparent in the cellobiose agar cultures, a period of 4 or 5 days incubation being required, a colony was picked and diluted serially into similar medium. After at least two transfers on cellobiose agar, in which only one colony type was observed in tubes of high dilution, the culture was considered to be pure. The cellulolytic activity of the pure culture was verified by inoculating liquid and solid cellulose media with a single colony from the cellobiose culture. Liquid dilution series and parallel agar series showed cellulose digestion at about the same dilutions. Pure cultures of the cellulose-digesting cocci were also obtained from liquid cellulose cultures inoculated with high dilutions of caecal contents. Stock cultures of the caecal cocci were maintained on cellulose agar. Cultures used in physiological studies were obtained by inoculating cellulose liquid media with colonies picked from cellobiose agar.

Characteristics of the isolated cocci

Morphologically, the caecal cellulolytic cocci isolated on seven separate occasions were similar. On primary isolation and in liquid cultures they appear as short-chain streptococci or diplococci. After prolonged cultivation on solid media the majority of the cells are in diplo- or micrococcus arrangements. Young cultures (48 hr.) are Gram-positive, but as the cultures age, the cells become Gram-variable. The cells of both old (7 days) and young cultures are iodophilic.

In primary cultures of the caecal cocci more rapid growth occurs in liquid than in solid cellulose media. In liquid cultures an incubation period of 7–20 days at 38° is necessary for complete disappearance of cellulose in the highest dilutions showing cellulose digestion. In agar cultures 23–78 days incubation is required before distinct zones of clearing, indicating cellulose digestion, are detected. Pure cultures of the cocci digest cellulose more rapidly than mixed cultures. An appreciable digestion of cellulose in liquid cultures occurs as soon as 24 hr. after inoculation and in solid media diffuse zones of clearing can be noted after 4 or 5 days' incubation. Pure cultures inoculated into cellobiose agar show colonies after 48 hr. incubation. The subsurface colonies on this medium are less wrinkled and larger than those on cellulose agar. No pigment has been observed.

The isolated caecal cocci are strict anaerobes, no growth being observed in cultures in which resazurin is not reduced to the colourless form. The optimum temperature for growth is 38–40°. No growth occurs at 45° and only very slow growth at 32°. Maximum decomposition of cellulose is evident in cultures in which the pH value is adjusted within the range of 6·6–7·1.

Nutrient requirements. Various substrates were tested for their ability to support growth of the caecal cocci in a medium which contained neither rumen fluid nor caecal extract. The following substances were tested: ascitic fluid (Difco), peptone (Difco), yeast extract (Difco), phytone (Baltimore Biological Laboratories; BBL), and trypticase soy broth (BBL). These substances were tested singly and in various combinations and, in some instances, rabbit serum in a concentration of 2 % (v/v) was also added to the medium. A medium containing 0·1 % (w/v) phytone, yeast extract, peptone and 2 % (v/v) rabbit serum plus the basal inorganic salt solution supported growth when inoculated with 0·2 ml. from the rapidly growing liquid cellulose culture. However, subcultures from these tubes to a similar medium failed to show growth. When this medium was inoculated with a single colony from cellobiose agar no growth occurred, although a 5 % (v/v) rumen fluid medium inoculated in a similar manner showed active cellulose digestion after 5 days' incubation. Ability of the phytone + yeast extract + peptone + serum medium to support growth in the initial culture was attributed to the rumen fluid carried over in the liquid inoculum. No substitute for rumen fluid or caecal extract has been found to support consistent growth of the caecal cellulolytic cocci.

Carbohydrate utilization. Ability of the caecal cocci to utilize various carbohydrates was tested. Sterile basal medium consisting of inorganic salt solution ($NaHCO_3$ decreased to 0·1 %) and 30 % (v/v) rumen fluid was inoculated with a 48 hr. liquid cellulose culture, the amount of inoculum being sufficient to give a final concentration of 0·5 % (v/v). A gas mixture of 95 % N_2 and 5 % CO_2 was bubbled through the medium at the time of transferring in order to maintain anaerobic conditions. The inoculated medium was transferred to sterile tubes and the carbohydrate solutions were added to each tube in sufficient quantities to give a concentration of 0·25 %. All of the carbohydrate solutions were sterilized by filtration. One set of tubes was incubated at 38°, and another set held at refrigerator temperature to serve as controls. Basal medium containing no carbohydrate, but inoculated and incubated, served as a control for possible fermentable substances present in the rumen fluid. Utilization of the substrate was determined by turbidity and acid production. In addition to cellulose the following carbohydrates were tested: rhamnose, L-arabinose, L-xylose, D-xylose, fructose, glucose, mannose, galactose, trehalose, cellobiose, sucrose, maltose, lactose, raffinose, dextrin, inulin, starch, glycerol, salicin, dulcitol, sorbitol and inositol. Of these substrates only cellobiose was utilized.

Fermentation products

Culture methods. Culture techniques used were similar to those employed by Hungate (1944). The culture flasks were of 100 ml. capacity with a glass sealed inlet and outlet. The basal culture medium contained inorganic salts (see culture methods) plus 20 % (v/v) rumen fluid. Filter-paper cellulose, in the form of a fine suspension, was added in concentrations of from 0·1 to 0·3 % (w/v); cellobiose was added in a concentration of 0·1 % (w/v). L-Cysteine monohydrochloride (0·4 %) was used as reducing agent. During the preparation and inoculation of the medium anaerobic conditions were maintained by the passage of oxygen-free gas through the medium. Nitrogen was used in all experiments with the exception of the cellobiose experiments in which CO_2 was used. The inoculum was 2–5 ml. of a 24 or 48 hr. liquid cellulose culture/50 ml. culture medium. The medium, after inoculation, was transferred aseptically to the culture flasks through the inlet side arm which was then sealed in a flame. The outlet arm was attached by means of pressure tubing to a vacuum pump and evacuated until the medium boiled vigorously. The outlet was then sealed in the flame. Incubation was for 10 days or 2 weeks at a temperature of 38°. Control flasks were similar to the experiment except that they were held at refrigerator temperature. After incubation, the flasks, control and experimental, were analysed for CO_2, H_2, volatile acids, non-volatile acids, ethanol, glycerol and reducing substances.

Analytical methods. The amount of cellulose fermented was determined by the dry-weight difference between the residue of the experimental and control flasks. Gas analyses were done with the Haldane apparatus. The dissolved CO_2 was liberated by acidification of the culture with dilute sulphuric acid and absorbed by ascarite. The quantity of gas liberated was determined by the weight increase of the ascarite absorption tubes. Ethanol was determined by dichromate oxidation of the neutral distillate. The acid produced from the oxidized ethanol was removed by steam distillation and the nature and quantity determined by a Duclaux distillation. The volatile acids were separated by partition chromatography in a silica gel column (Elsden, 1946) and the quantity of each estimated from Duclaux distillations. Succinic acid was determined manometrically with a succinoxidase preparation from mammalian heart muscle (Umbreit, Burris & Stauffer, 1945). The method of Friedemann, Cotonio & Shaffer (1927) was used to determine lactic acid. Qualitative tests for reducing substances were negative.

Results. Analyses were run in duplicate, a control flask being run simultaneously with its corresponding experimental flask. Gas analyses were performed only for those experiments in which cellulose was the substrate. Results obtained in all experiments were essentially the same, and no significant differences were observed between the products formed from cellulose and those from cellobiose. The fermentation products included ethanol, acetic acid, succinic acid, a trace of formic acid, CO_2 and H_2. In one cellulose experiment lactic acid was produced. Propionic and butyric acid were never found in concentrations significantly greater than in the controls. Tests for glycerol (Amerine & Dietrich, 1943) were negative in two separate experiments. Results of a typical cellulose fermentation analysis are expressed in Table 1. A comparison of the non-gaseous fermentation products of cellulose and cellobiose are found in Table 2.

Table 1. *Cellulose* fermentation products of caecal coccus strain F-6*

End-products	Cellulose control (mg. atoms C)	Cellulose experimental (mg. atoms C)	Difference (mg. atoms C)
Ethanol	—	0·3	0·3
Formic acid	—	Trace	—
Acetic acid	1·32	1·62	0·3
Propionic acid	0·50	0·51	0·01
Butyric acid	0·42	0·40	—
Lactic acid	0·08	0·08	—
Succinic acid	—	0·23	0·23
CO_2	0·007	0·54	0·533
H_2†	—	—	—

* Cellulose utilized, 51 mg. † 0·20 mmol.

Table 2. *Non-gaseous fermentation products of cellulose* and cellobiose* by caecal coccus strain F-6*

End-products	Cellulose (mg. atoms C)	Cellobiose (mg. atoms C)
Ethanol	0·55	0·45
Formic acid	Trace	Trace
Acetic acid	0·26	0·34
Propionic acid	—	—
Butyric acid	—	—
Succinic acid	0·71	0·64

* Substrate utilized: cellulose 94 mg., cellobiose 100 mg.

DISCUSSION

Cellulolytic cocci can be consistently isolated in significant numbers; 500 thousand to 72 million/g. moist caecal contents, from domestic rabbits fed diets of dehydrated alfalfa hay, Larro pellets,‡ Friskies dog food§ plus oats, or cracked barley plus alfalfa hay (Table 3). Successful isolations of the caecal cellulolytic cocci have been made on seven separate occasions during a period of 2½ years. The rabbits from which these isolations were made came from different sources and were obtained at different times throughout the experimental period. These facts suggest that the cellulose-digesting cocci were not chance inhabitants of the adult rabbit caecum but rather members of the normal microflora (Table 3).

‡ Larro Green Pellets for Rabbits, minimum per cent: crude protein, 15; crude fat, 2; crude fibre, 19; crude ash, 7·75; moisture, 12; minerals, 1. General Mills Inc., Sperry Division, San Francisco, California.

§ Friskies Dog Food, Albers Milling Co., Los Angeles, California.

Table 3. *Numbers of caecal cocci demonstrated from rabbits fed various diets*

Diet	Number of caecal cellulolytic cocci/g. moist caecal contents
Larro pellets	64 million
Larro pellets	45 million
Larro pellets	25 million
Larro pellets plus grass cuttings	72 million
Friskies plus oats	22 million
Cracked barley plus alfalfa hay	6 million
Dehydrated alfalfa hay	500 thousand
Cracked barley*	—

* Seven unsuccessful isolation attempts.

Failure to isolate cellulose-digesting bacteria from the caecal contents of rabbits maintained on a cracked barley diet may have been due to inadequate culture techniques rather than to an absence of these organisms. These isolations were attempted at a time when the culture procedure had not yet been perfected and negative results are therefore not conclusive. However, diet may affect the numbers of cellulolytic bacteria in the rabbit caecum. Recent investigations of Baker, Nasr, Morrice & Bruce (1950) indicate that the only carbohydrate fractions of cracked barley to reach the caecum of the rabbit would be those, such as the 'hemicelluloses' and 'celluloses', which resist the action of digestive enzymes of the rabbit. Analyses of the food and faeces of rabbits which had been maintained for 3 weeks on a cracked barley diet showed a very small amount of 'cellulose' in cracked barley and no utilization of this 'cellulose' by the rabbit. In fact, the weight of the cellulose fraction in the faeces of this animal was slightly higher than in the food consumed. The small quantity of 'cellulose' in cracked barley and the apparent inability of the rabbit to utilize this 'cellulose' suggests that a barley diet would not support a large cellulolytic flora in the caecum. Utilization of added cotton cellulose by rabbits fed the cracked barley + filter-paper diet ranged from 24 to 34 % (Table 4).

Direct microscopic examination of caecal contents used in culturing showed Gram-positive iodophilic cocci similar to those isolated in culture and to those referred to by Baker & Martin (1937) as being active in the decomposition

Table 4. *Comparison of the quantity of food fractions eaten with the quantity excreted*

	Rabbit I, cotton cracked barley diet			Rabbit III, cotton cracked barley diet			Rabbit IV, cotton cracked barley diet			Rabbit II, cracked barley diet		
	Food (g.)	Faeces (g.)	Diff. (g.)	Food (g.)	Faeces (g.)	Diff. (g.)	Food (g.)	Faeces (g.)	Diff. (g.)	Food (g.)	Faeces (g.)	Diff. (g.)
Weight	1050	367	683	1049	385	664	925	313	612	792	152	640
Wt. sol. alco.-benzol diastase-water	541	23	518	540	26	514	476	19	457	462	20	442
Wt. sol. 2 % H_2SO_4 'hemicellulose'	79	39	40	79	45	34	69	29	40	190	20	170
Wt. sol. 72 % H_2SO_4 'cellulose'	353	252	101	352	267	85	311	205	106	66	82	−15

of cellulose in the rabbit caecum. Direct counts of these cocci indicated they were present in higher numbers than were demonstrated by culture methods. Between 112 million and 2 billion (2×10^9) diplococci/ml. caecal contents were shown to be present as a result of direct counts, whereas the parallel culture counts showed 25–72 million diplococci/g. moist caecal contents. This discrepancy may be due to the presence of additional species of cocci having the same size and staining reactions as the cellulolytic coccus. However, the numbers of micro-organisms demonstrated by cultures are usually significantly less than those obtained by direct counts. This would be particularly true for cellulolytic organisms such as the coccus, which tend to cling to undigested particles in the caecum.

The products formed from cellulose fermentation by the caecal cocci are ethanol, acetic acid, succinic acid, a trace of formic acid, CO_2 and H_2. Acetic and succinic acid are found in highest concentration and approximately equal quantities. Of these non-gaseous fermentation products acetic acid has been reported as being present in rabbit caecal contents.

Analyses of rabbit caecal contents for volatile fatty acids by Elsden *et al.* (1946) showed the presence of acetic, propionic and butyric in the following percentages of the total fatty acid content: acetic, 78·25; propionic, 9·3; butyric, 12·45. The production of significant amounts of acetic acid by the cocci isolated from the caecum favours the conclusion that the isolated caecal coccus is an important cellulose digester in the rabbit caecum. The difference between the products formed from the fermentation of cellulose by pure cultures of the caecal coccus and those occurring normally in the caecum may be attributed, in part, to the mixed flora and substrate of the caecum.

The relationship of the caecal cocci to previously described anaerobic cellulose-digesting cocci is interesting. Except for the absence of yellow pigment it is quite similar to *Ruminococcus flavefaciens* as described by Sijpesteijn (1951). Many of the characteristics of the caecal cocci resemble those of the rumen cocci (Hungate, 1950). Morphologically these organisms are quite similar. Most of them appear to be Gram-variable; young cultures show many Gram-positive cells, but older cultures show a predominance of Gram-negative forms. The loss of ability to retain the Gram stain may be influenced by the accumulation of fermentation products. On primary isolation the caecal cocci, as well as the rumen cocci, occur in long or short chain arrangements. After repeated subcultivation, especially on agar media, the organisms resemble micrococci. The rumen and caecal cocci are obligate anaerobes which fail to grow in a medium in which resazurin is not reduced to the colourless form. The majority of the rumen cocci and the caecal cocci utilize only cellulose and cellobiose as substrates. Also, many strains of these organisms will not grow in a medium that does not contain rumen fluid. With a few exceptions, fermentation products of the rumen and caecal cocci are similar. The morphological, cultural and biochemical similarities of these anaerobic, cellulolytic cocci suggest that they form a closely related group of bacteria.

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REFERENCES

AMERINE, N. A. & DIETRICH, W. C. (1943). Glycerol in wines. *J. Ass. off. agric. Chem., Wash.* **26**, 408.

BAKER, F. & MARTIN, R. (1937). Some observations of the iodophile microflora of the caecum of the rabbit; with special regard to the disintegration of cell wall substances. *Zbl. Bakt.* (2 Abt.), **96**, 18.

BAKER, F., NASR, H., MORRICE, F., & BRUCE, J. (1950). Bacterial breakdown of structural starches and starch products in the digestive tract of ruminant and non-ruminant mammals. *J. Path. Bact.* **62**, 617.

ELSDEN, S. R., HITCHCOCK, M. W. S., MARSHALL, R. A. & PHILLIPSON, A. T. (1946). Volatile acid in the digesta of ruminants and other animals. *J. exp. Biol.* **22**, 191.

ELSDEN, S. R. (1946). The application of the silica gel partition chromatogram to the estimation of volatile fatty acids. *Biochem. J.* **40**, 252.

FRIEDEMANN, T. E., COTONIO, M. & SHAFFER, P. A. (1927). The determination of lactic acid. *J. biol. Chem.* **73**, 335.

HUNGATE, R. E. (1944). Studies on cellulose fermentation. I. The culture and physiology of an anaerobic cellulose-digesting bacterium. *J. Bact.* **48**, 499.

HUNGATE, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bact. Rev.* **14**, 1.

SIJPESTEIJN, A. K. (1951). On *Ruminococcus flavefaciens* a cellulose-decomposing bacterium from the rumen of sheep and cattle. *J. gen. Microbiol.* **5**, 869.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Minneapolis, U.S.A.: Burgess Publishing Co.

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Ainsworth, G. C. (1952). *J. gen. Microbiol.* 7, 358–361.

The Incidence of Air-borne *Cladosporium* Spores in the London Region

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SUMMARY: Spore trapping by the Petri dish method showed the incidence of air-borne *Cladosporium herbarum* spores, which fluctuated markedly from day to day, to be similar in Central London and in an outer suburb throughout the year.

Although it was in Europe that the ability of air-borne fungus spores to act as allergens was first recognized, the most detailed surveys of the fungus spore content of the air have been made in the United States. Increasing attention is, however, being paid to this topic elsewhere, and in Britain the Asthma and Allergy Unit at Cardiff is now supplementing studies on the incidence of air-borne pollen with investigations on the occurrence of fungus spores. Reports have already appeared on the incidence of *Alternaria* spores (Hyde & Williams, 1946) and of fungus spores in general (Hyde & Williams, 1949).

In 1946, during a search for new antibiotics, it was decided to follow the clue provided by Fleming's discovery of penicillin and to sample the fungus spores present in the atmosphere, but by design, and this note summarizes certain data obtained by the systematic exposure of plates in and around London between August 1946 and September 1948.

METHODS

As a result of experiments with different media and with different periods of exposure at different times of day of varying numbers of plates freely exposed to the air and variously protected from rain, it was decided to sample by exposing six 9 cm. Petri dishes of potato glucose agar for 5 min., protected from rain in an apparatus, of similar type and dimensions to that described by Hyde & Williams (1945), set up 10 ft. from the ground in a grass field well away from the laboratory buildings. Exposures were made at 12 noon on 6 days each week and the plates were subsequently incubated at 25°, the number of colonies developing being counted on or about the 7th day. The colonies were divided into two categories, 'bacteria and yeasts' and 'fungi'.

In addition to the exposure of plates, a microscope slide smeared with glycerine jelly was exposed in the tower for 24 hr. (noon–noon) every day. Rain and the daily maximum and minimum temperatures were recorded, and the direction of the wind at the time of exposure of the plates was noted.

The exposures were made at Sutton, Surrey, situated about 15 miles south-west of London at an altitude of 250 ft.

RESULTS

Effect of falling rain

In the course of preliminary experiments a series of thirty-six exposures was made during August 1946. On thirty occasions the weather was fine, on six rain was falling and the number of fungi developing on plates exposed during rain greatly exceeded that during dry periods (av. wet, 189; av. dry, 43). On one occasion when the plates were exposed during a sharp thunder storm the number of colonies developing on the six plates was 565, the highest recorded during the survey. The results suggested that this increase in the spore content of the air was only temporary and that the number soon fell to 'normal' when the rain stopped, but the increase during rain was unexpected and did not appear to have been noted by modern observers.

The results obtained in 1872 by D. D. Cunningham, during what may be regarded as the first scientific study of the spore content of the air by the 'vertical slide' method, show a similar effect in that at the Presidency Jail, Calcutta, and at Alipore, more spores were trapped on days for which rain was recorded than on dry days; the averages for the 24 hr. periods being: Presidency Jail, dry 145, wet 165; Alipore, dry 132, wet 197 (Cunningham, 1873; data in Tables 1 and 2).

A possible explanation of this effect is that the rain-drops wash spores out of the upper air and increase the turbulence of the air near the ground. No relation was noted between periods of wet weather and the daily fluctuations in the numbers of spores trapped (see below).

Effect of the time of day

During the same series of experiments, plates were exposed at 10 a.m., 12 noon, 2 p.m. and 4 p.m. on 6 fine days in August 1946 when the average number of spores trapped at the different times were 40·8, 39·3, 40·2 and 54·7, respectively. This suggested an increase during the afternoon, probably due to a greater turbulence of the air, and it was for this reason that noon was selected for routine sampling.

Daily fluctuation in the number of Cladosporium *colonies*

It was not possible to identify all the fungi on all the plates and *C. herbarum*, a species known to be involved in allergic disorders, is the only fungus for which an accurate record is available for the whole series. *C. herbarum*, an unspecialized and ubiquitous saprophyte, accounted for approximately half of the 'fungus' colonies and this is in accord with the findings of Hyde & Williams (1949) for Cardiff. *C. herbarum* was also the species most frequently recorded from the air by Blackaller (1950) in Mexico and by Ambler & Vernon (1951) in New Zealand.

The daily incidence of *Cladosporium* during October–November 1946 and January–February 1947 is summarized in Fig. 1. The shape of the graph is very similar to that for all 'fungi' during the same period (see Ainsworth, 1952, fig. 5), and is characterized by constant fluctuations. The reasons for these fluctuations are obscure. There would appear to be no obvious correlation with periods of wet weather. There is, however, a general fall in numbers during the winter and particularly during periods of snow. The only day on which the plates remained sterile during the 5 min. exposure was on 24 February 1947 when snow covered the ground.

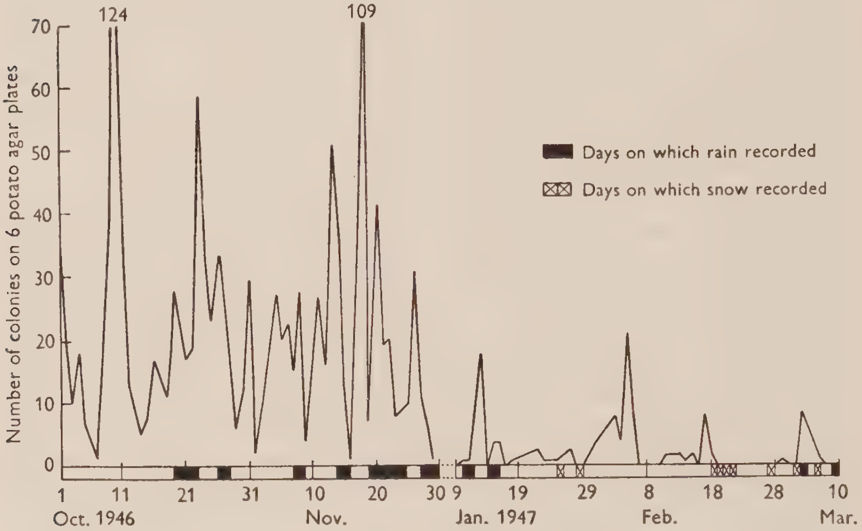

Fig. 1. Daily incidence of *C. herbarum* spores trapped at Sutton, Surrey.

Comparison of the incidence of Cladosporium *spores in central London and in an outer suburb*

When the total numbers of fungi trapped were correlated with the direction of the wind the average number of colonies per exposure was: N. (NW., N., NE.), 29·4; S. (SW., S., SE.) 45·7; E. (NE., E., SE.), 34·4; W. (NW., W., SW.), 39·0. This suggested that fewer colonies developed when the wind had traversed the metropolitan area than when it had blown over the open country to the south and west, and so in the following year an opportunity was taken to compare central London with Sutton.

The method was as described except that five malt agar plates were exposed at each sampling (the sixth being kept unopened as a control) and the exposures were made only once a week, simultaneously at 12 noon on the roof of the London School of Hygiene and Tropical Medicine (London, W.C. 1) and through the skylight of a house in Sutton a few hundred yards from the site of the 1946–7 exposures.

As in the earlier series, *C. herbarum* was the only fungus consistently identified throughout the experiment, and the average monthly incidence of this species at the two stations is summarized in Fig. 2. The two curves show very close agreement and suggest that 'local' conditions have little effect on the general fungus spore content of the outdoor air. Both curves also illustrate the seasonal cycle in the incidence of *Cladosporium* spores which has a maximum in the summer and a minimum during the winter months as noted by Hyde & Williams (1949).

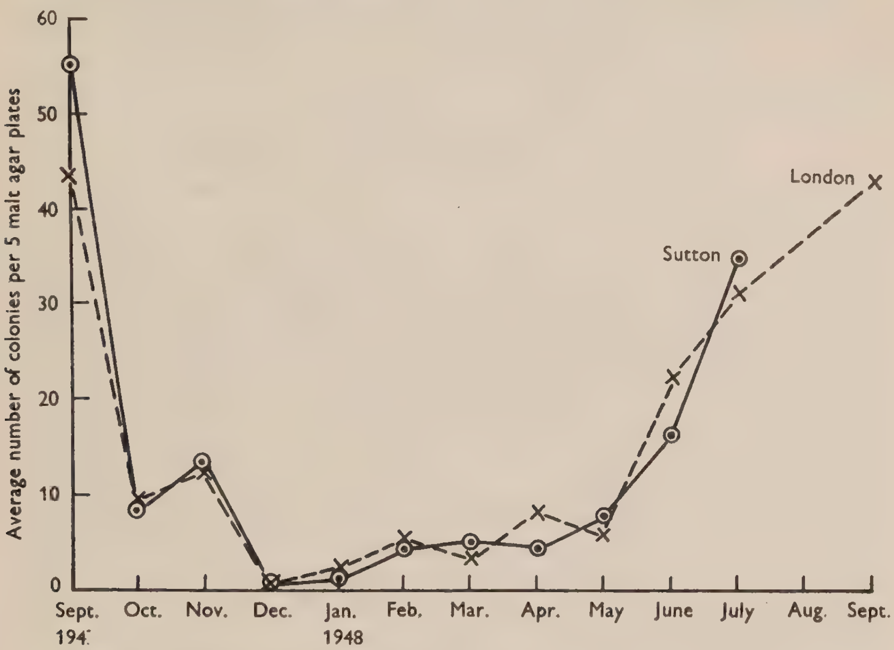

Fig. 2. Average monthly incidence during 1947–48 of *C. herbarum* spores trapped at Sutton, Surrey and in Central London.

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REFERENCES

Ainsworth, G. C. (1952). *Medical Mycology. An Introduction to its Problems.* London: Sir Isaac Pitman and Sons Ltd.

Ambler, M. P. & Vernon, T. R. (1951). Airborne mould spores. A survey of the atmospheric spore load in the Auckland City and suburban area. *N.Z. J. Sci. Tech.* A, **33**, 79.

Blackaller, F. A. (1950). Estudio de los hongos atmosféricos en la ciudad de Guadalajara, Jalisco. *Medicina, Méx.* **30**, 111.

Cunningham, D. D. (1873). *Microscopic Examinations of Air.* Calcutta.

Hyde, H. A. & Williams, D. A. (1945). Studies in atmospheric pollen. II. Diurnal variation in incidence of grass pollen. *New Phytol.* **44**, 83.

Hyde, H. A. & Williams, D. A. (1946). A daily census of *Alternaria* spores caught from the atmosphere at Cardiff in 1942 and 1943. *Trans. Brit. mycol. Soc.* **29**, 78.

Hyde, H. A. & Williams, D. A. (1949). A census of mould spores in the atmosphere. *Nature, Lond.* **164**, 668.

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Hewitt, L. F. (1952). *J. gen. Microbiol.* 7, 362–371.

Diphtheria Bacteriophages and their Relation to the Development of Bacterial Variants

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SUMMARY: Many strains of *Corynebacterium diphtheriae* carry phages capable of lysing other strains. Diphtheria phages are type-specific and do not lyse strains which are serologically unrelated but susceptibility to a phage may vary among different members of the same serological type and this can be used to differentiate subtypes of different biological behaviour. Phage-resistant mutants are readily selected in diphtheria cultures and the resistant mutants frequently have characteristics quite distinct from those of the parent strain, e.g. virulent organisms have been isolated from a number of avirulent strains. It is suggested that the possibility of converting avirulent strains to virulent ones may be of importance epidemiologically and that the phage selection of mutants may account for the wide variety of serological types isolated from patients. Some strains found to be resistant to type-specific phages have been shown to be lysogenic and when subjected to ultra-violet irradiation liberate phage particles which are capable of lysing susceptible strains of the same serological type. The diphtheria phages investigated are thermolabile, difficult to filter, of fairly large size and give rise to small plaques of lysis with susceptible strains.

Strains of *Corynebacterium diphtheriae* have been classified into some sixty serological types by agglutination methods (Hewitt, 1947, 1948*a*), and the literature detailing the variations in virulence, colonial form, fermentation reactions, etc., is too vast for brief review, but the origin of these wide variations and their effect on the epidemiology of diphtheria remain obscure. It is found that antibiotics such as penicillin and streptomycin alter the characteristic properties of diphtheria strains by the selection of resistant mutants (Hewitt, 1948*b*), but these drugs are unlikely to have played any important part in the evolution of the strains encountered in infections. One purpose of this communication is to investigate the possibility that bacteriophages may be the natural agents responsible for the great variety of strains that are isolated from diphtheria patients and carriers.

Phage typing for the identification of infecting strains has become an established procedure in the case of a number of organisms (Burnet, 1930; Craigie & Yen, 1938; Felix & Anderson, 1951; Wilson & Atkinson, 1945; Boyd, 1950), but although the occurrence of phages active against *C. diphtheriae* has been described by a number of workers (Blair, 1924; Smith & Jordan, 1931; Stone & Hobby, 1934; Keogh, Simmons & Anderson, 1938; Toshach, 1950; Freeman, 1951; Parsons & Frobisher, 1951; Freeman & Morse, 1952) there is insufficient evidence available to judge whether *C. diphtheriae* can be classified on the basis of phage sensitivity or whether diphtheria bacteriophages are of common occurrence. An investigation into the natural distribution of diphtheria bacteriophages, their properties and specificity is therefore necessary.

MATERIALS AND METHODS

Sources of strains. The strains of *C. diphtheriae* used were in some cases freshly isolated from patients in various parts of the world (Great Britain, continental Europe, the Middle East, Africa, Australia and America), and others which had been kept in laboratory cultivation. Methods of isolation have been described previously (Hewitt, 1947).

Serological typing. Anderson, Happold, McLeod & Thomson's (1931) heated blood tellurite agar medium was used for the characterization of the Mitis, Intermedius and Gravis groups, and serological typing was by agglutination with rabbit sera as described previously (Hewitt, 1947, 1948*a*).

Virulence and toxigenicity tests. The virulence of strains was tested by the lethal effect of cultures washed off Loeffler slopes and injected subcutaneously into guinea-pigs. The toxigenicity was determined by growing the strains in a standardized papain digest broth and titrating the toxin in the filtrate by a modification of the Römer (1909) intracutaneous test as described previously (Hewitt, 1948*a*).

Isolation of phages. The phages investigated were isolated from phage-carrying diphtheria strains. As described in a later section ultra-violet irradiation was used to demonstrate the lysogenicity of some strains, but in other cases it was possible to detect lysis when a culture was 'spotted' on an agar plate already inoculated with another strain, as found by Fisk (1942) in the case of staphylococcus phages. For the isolation of some phages mixed cultures of two diphtheria strains in broth were incubated and the filtrate was added to a culture of the strain for which the phage was sought. Several repetitions of this process were generally necessary before appreciable phage activity could be detected.

Phage titrations. The lysis of broth cultures was frequently a poor guide to phage activity, but areas of lysis on solid media proved a convenient index of phage potency. Broth cultures of the susceptible strain were streaked on nutrient agar plates and loopfuls of suitable phage dilutions were spotted on the streaks. Dilutions were made in broth in order to avoid variation in the concentration of cations or other broth constituents. Enumeration of phage particles by the counting of plaques on solid media by the method of Gratia (1936) is not practicable with many diphtheria strains, since growth is not confluent but tends to occur in clumps which obscure the plaques.

RESULTS

Phage-carrying strains. Of the first eight diphtheria strains examined at random five readily yielded phages. Two of the phage-carrying strains belonged to the Mitis group, one was Gravis and the other two Intermedius. The phages were, as shown later, highly specific in their range of activity, but phages of widely different host specificity can be isolated from a single lysogenic strain. Thus from one lysogenic Intermedius strain three phage filtrates were obtained by subculturing in the presence of a Gravis type I strain, a Mitis type X strain and an Intermedius strain respectively. The phages obtained each lysed only the homologous strain and other strains of the same serological type. This, however, does not necessarily mean that the lysogenic strain was carrying a number of different phages but may have been due to adaptation of the phage to the susceptible strain during the course of isolation.

Adaptation of phage. When a phage for any particular serological type was required it was obtained either by culturing together a strain of this type with a phage-carrying strain, or by adapting one of the phages already obtained to the new type. This adaptation was effected by adding phage to a broth culture of the strain, incubating for 18 hr. and adding the supernatant fluid to a fresh subculture of the strain. If no effective phage had appeared after six subcultures the experiment was generally abandoned and a fresh combination of phage and strain was investigated in the same way. By this method thirty-six bacteriophages effective against twenty-four serological types have been obtained, and more than eighty strains have been found to be lysed by one or other of the phages.

Heat lability of phage. Diphtheria phages were completely destroyed by heating at 55° for 30 min. but survived at 50°; *C. diphtheriae* cultures were sterilized by heating at 50° for 30 min. but survived at 46°. Using this difference in heat stability it is possible to rid phage suspensions of living diphtheria bacilli by first centrifuging and then heating at 50°, but chance bacterial contaminants introduced during centrifuging operations, etc., may survive heating at 50°, and filtration yields a clearer product.

Filtrability of phage. Filtration of phage through a Seitz or Ford sterilizing pad or a Pasteur-Chamberland L3 candle removed lytic activity. Sterilization without serious loss of activity was effected, however, by filtration through Gradocol membranes of average pore diameter (A.P.D.) between 0·5 and 0·7 μ.

Size of phage particles. The plaques produced by diphtheria phages are small, generally not exceeding 0·3 mm. in diameter. Elford & Andrewes (1932) point out that the formation of small plaques is indicative of large phage particles, and this is in accord with the observations mentioned in the previous section that phage activity is readily lost on filtration. Using Gradocol membranes of different porosity it was found that some activity passed through membranes of 0·115 μ. A.P.D. but all lytic activity was held back by membranes of 0·075 μ A.P.D., the lytic activity being recovered by washing the membranes with broth. This evidence suggests a particle size of about 60–80 mμ.; the phages examined by Elford & Andrewes (1932) ranged in size from the smallest of 8–12 mμ. to the largest of 50–75 mμ. It appears, therefore, that diphtheria phages are of comparatively large size, and further work is being carried out to define the particle size more closely.

Relation of phage-susceptibility to serological specificity. Thirty-six phages have been investigated, and these have been found to be type-specific, each being active only against strains of a single serological type; but, as shown below, the degree of specificity of phage-susceptibility is higher than that of serological specificity and in some cases the phages revealed differences between strains of the same serological type. So far, phages are available for twenty-four different serological types, and a few examples are quoted to illustrate the degrees of specificity observed.

In the case of Gravis type I strains five phages have been prepared. These phages lysed a number of Gravis type I strains but no Mitis or Intermedius strains, nor any Gravis strains which were not serologically related. The phage titres were not, however, the same when tested against different Gravis type I strains, some of which showed varying degrees of resistance to one or other of the phages. Results with three contrasting Gravis type I strains are shown in Table 1.

Table 1. *Titres of phage activities against different Gravis type I strains*

	Strain		
Phage	A	B	C
1	100	1000	10
2	1000	100	0
3	100	0	1
4	1000	1	1
5	1000	10	1

Titres are expressed in terms of the dilution of phage of which one loopful produced confluent lysis on a culture of the strain on an agar plate.

The explanation of these differences in susceptibility to phage among members of a single serological type is discussed in a later section where the lysogenicity of strain C is demonstrated.

In the case of a Gravis type II phage only the homologous strain was lysed. When adapted by subculture with another Gravis II strain its range of activity was widened, so that it lysed six Gravis II strains, but as some strains of this type were still not susceptible it was further adapted to one of the non-susceptible strains; this further adaptation decreased the lytic activity against some of the previously susceptible strains. Thus the phages are type-specific but further degrees of specificity are shown in the activity of some phages. This is clearly seen in the case of phages prepared against a Mitis type III strain.

All Mitis type III strains are agglutinated by the same agglutinating rabbit serum prepared by intravenous injection of a killed vaccine, but of twenty-four strains examined, only twelve were susceptible to the type-specific phage, the others being completely resistant. The two subtypes were quite clearly differentiated, the resistant strains being virulent and toxigenic, whilst the susceptible strains were found to be avirulent and non-toxigenic. Further implications of this phage differentiation of subtypes become apparent in the next section dealing with phage-resistant mutants.

Selection of phage-resistant mutants. Addition of the appropriate phage to broth cultures of susceptible diphtheria bacilli has in no case been found to produce complete clearing but, after incubation, resistant mutants grow out and these on subculture are found to be no longer susceptible to the phage. The phage-resistant mutants, however, sometimes differ from the parent strain in respects other than that of phage-susceptibility. Thus in the case of a Gravis type VIII strain and the appropriate phage, the phage-resistant culture was virulent and toxigenic, whilst the original strain was avirulent.

An interesting example of this difference in properties is in the case of Mitis type III strains. As mentioned in the previous section, the phage-susceptible strains are avirulent. Phage-resistant mutants isolated from six of these strains proved to be virulent and toxigenic. Through the agency of phage, therefore, the avirulent strains had been converted to the virulent subtype.

Heterogeneity of resistant mutants. It is noteworthy that the virulent phage-resistant mutants selected by the action of bacteriophage on the avirulent Mitis III strains described in the previous section were not all of equal toxigenicity. The titre of toxin produced in the standardized papain digest broth varied in the case of the different strains from 0·05 to 4·0 units/ml. Moreover, in each case by picking colonies on solid media, phage-resistant mutants remaining avirulent and non-toxigenic could be selected. Differences in the properties of phage-resistant mutants were also found in the case of phage-resistant mutants of a Gravis type I strain (strain A in Table 1). In one experiment phage was added to a culture of the strain on an agar plate, and, after incubation, an area of confluent lysis appeared where the phage had been added. There were, however, a few colonies of resistant mutants. Five of these were picked off with a platinum wire and subcultured separately. Apart from differences in growth characters, granularity of growth, etc., the toxigenicity of the mutants under standardized conditions varied from 0·01 to 0·2 unit/ml. It becomes clear, therefore, that all the mutants selected by means of their resistance are not identical.

Further evidence of this is seen in the degree of resistance of different mutants to various phages. Examples of these differences among wild strains are given in Table 1, and it is now possible to consider the properties of phage-resistant mutants obtained in the laboratory. Again working with the Gravis type I, strain A, mutants were prepared against each of the five Gravis type I phages previously mentioned, and the susceptibility of each resistant mutant to the same phages was tested. The results are summarized in Table 2. Resistant strains are designated by the phage to which they were made resistant, e.g. R1 signifies the mutant resistant to phage no. 1.

The mutants R1 and R3 which were still susceptible to phage 2 were now subjected to the action of this phage, and the resistant mutants R1, 2 and R3, 2 were isolated, but these were found to be resistant to all five phages. The experiments summarized in Table 2 show that it is possible to produce in the laboratory strains of varying phage susceptibility similar to those isolated from diphtheria patients and carriers.

Lysogenicity and ultra-violet irradiation. It has just been shown that it is possible to produce in the laboratory mutant strains of varying phage susceptibility, and it seemed possible that the similar range of susceptibility encountered among strains in the field might reflect the phage history of such strains. Further evidence was sought, therefore, that diphtheria strains showing resistance to type specific phages had encountered phages at some stage in their history.

Evidence of this was found in the case of Gravis type I strains. Strain A, as mentioned previously, is susceptible to lysis by five type-specific phages, whilst strains C and D show varying degrees of resistance. Cultures of strain A were streaked on agar plates and strains C and D were spotted on these streaks. When the plates were incubated little evidence of lysis was seen, but if the plates were first subjected to ultra-violet irradiation definite evidence of lysogenicity was obtained. The induction of phage liberation by ultra-violet irradiation has been described in the case of *Bacillus megaterium* by Lwoff, Siminovitch & Kjeldegaard (1950). It was found necessary to choose the dosage carefully, since too intense an exposure to ultra-violet light either sterilizes the cultures or results in so high a mortality rate that, instead of confluent growth of the susceptible strain, there are separate colonies obscuring areas of lysis. Under the experimental conditions used the appropriate exposure was for 10 sec. at a distance of 30 cm. from a mercury vapour lamp, but an attempt was not made to calibrate the intensity of radiation in terms of energy units. When the plates which had been irradiated under these conditions were incubated areas of lysis were seen where the phage-resistant strains had been applied. A portion of the lysed area of growth was transferred to a broth culture of the susceptible strain with a platinum wire. After incubation the supernatant fluid was filtered through a Gradocol membrane and repetition of the passage yielded a potent phage filtrate which lysed twenty-six other Gravis type I strains. It should be noted that the phage did not lyse the lysogenic strain from which it had been isolated, nor did it lyse two other resistant strains. Thus the strains resistant to the type-specific phage had been shown to be carriers of a phage active against susceptible strains of the same serological type. Direct evidence of previous contact with phage is therefore available.

Table 2. *Susceptibility of resistant mutants to other phages*

Phage no.	Strain no.				
	R1	R2	R3	R4	R5
1	0	100	0	0	0
2	100	0	100	0	0
3	0	100	0	0	0
4	0	100	100	0	0
5	100	100	100	0	0

Titres are given in terms of the dilution of the phage producing confluent lysis.

DISCUSSION

The hypothesis on which this work is based is that, in the case of diphtheria at any rate, bacteriophage may well be one of the principal natural agents responsible for controlling bacterial variation in infections. The means by which bacteria suddenly increase in virulence and epidemics occur is rather obscure for although, as with other living organisms, mutation among the bacteria has long been recognized, it has not been clear how whole cultures could rapidly undergo a permanent change in their characters. Adaptive changes are generally temporary and dependent upon environment, whilst the occurrence of one mutant bacterium in perhaps a million would not be expected to affect appreciably the properties of the culture as a whole, and the processes of natural selection would appear to be too slow to account for the rapid alterations encountered in infections. On the other hand, bacteriophage will lyse all the normal bacteria in a susceptible culture leaving intact only the few mutants resistant to the phage so that the resistant organisms that grow out will consist solely of mutants. Thus in a few hours the entire bacterial population changes from that of the parent strain to the mutant form. It thus seems at least plausible that bacteriophage may be of importance in epidemiology.

It was first necessary to establish that diphtheria phages occur naturally, and this was readily shown. The first eight strains examined at random were of six serological types, and five of these, including Gravis, Intermedius and Mitis strains, were found to be phage carriers, and potent phage filtrates have been obtained from each of them. Exhaustive surveys covering many strains from different sources would be necessary to give a statistically significant estimate of the frequency of occurrence of diphtheria phages, but it is already clear that they are of quite common occurrence.

It was found that the phages are type-specific and do not lyse strains which are not serologically related. The specificity is of a higher order than that seen in agglutination reactions, and many strains show varying degrees of resistance to their type-specific phages. There are several points of interest in this phage resistance. In the first place it was found that strains of one serological type, Mitis type III, were clearly differentiated into two subtypes according to their phage susceptibility. The phage-resistant strains were found to be virulent and toxigenic, whilst the susceptible strains were avirulent and non-toxigenic. Moreover, when some of the phage-susceptible avirulent strains were subjected to the action of phage and the phage-resistant mutants which grew out were isolated, they were found to be virulent and toxigenic. It had thus been possible in the laboratory to convert one subtype into the other and to transmute an avirulent into a virulent strain. Apart from the theoretical interest of these results the possible epidemiological significance is quite evident. In addition to providing an explanation of the variation in virulence of infecting organisms the experiment indicates how the carrier of an apparently harmless organism might become the disseminator of disease should an appropriate phage be present. At the present time the incidence of diphtheria in this country is unprecedentedly low and a high proportion of the child population has been rendered immune to diphtheria toxin, but there are still many carriers of the diphtheria bacillus. A strain received recently was isolated from a patient with sore throat and transient pyrexia. The child was in an orphanage in which the whole community had been fully immunized. The organism appeared to be typically *C. diphtheriae* but was quite avirulent and might well be dismissed as of no epidemiological significance. If, however, such strains in contact with phage can be transformed into virulent organisms they may prove the latent source of epidemics in the future, should the present trend of decline in immunization rate result in a susceptible population. It is of interest in this connexion to note the report of Edward & Allison (1951) that of forty-four strains of *C. diphtheriae* isolated from fully immunized persons nine were avirulent, and recently Bradshaw, Dixon, Mawson, Turner & Zinnemann (1952) reported the isolation of five avirulent strains of *C. diphtheriae* from school children in a highly immunized community. That this alteration of virulence in the presence of phage is not a fortuitous and rare phenomenon is suggested by the recent work of Freeman (1951), Parsons & Frobisher (1951) and Freeman & Morse (1952), who also report the conversion of avirulent strains to virulence after treatment with bacteriophage.

It is of interest to decide if there is any evidence that diphtheria strains isolated from infected patients have actually been subjected to the action of bacteriophage at any stage of their history, and whether phage has played any part in the evolution of the large number of serological types of *C. diphtheriae* encountered in field work. Indirect evidence is available in the varying phage-resistance of strains isolated from diphtheria patients and carriers. The case of Gravis type I strains can be taken as an example. Several type-specific Gravis type I phages of different origin have been isolated, and it has been found that the susceptibility of different Gravis type I strains to these phages varies widely. It seemed reasonable to conclude that the phage-resistant strains had developed resistance by means of mutant selection through contact with the appropriate bacteriophage at some stage of their history, and it remained necessary to show that such contact with bacteriophage had in fact occurred. The necessary evidence was forthcoming when it was found that ultra-violet irradiation induced the liberation from two phage-resistant Gravis type I strains of phage particles which lysed susceptible strains of the same serological type. The resistant strains could only have acquired such lysogenicity by previous contact with type-specific phage.

Of course, the fact that it may not be possible to demonstrate lysogenicity in all phage-resistant strains does not exclude the possibility that they have acquired such resistance after contact with bacteriophage, since not all phage-resistant mutants are lysogenic. Mutants with a slightly different surface structure fail to absorb phage and are not lysed. This second method of developing phage resistance may well be of great importance in the evolution of new serological types of bacteria, since only organisms differing from the rest of the culture will survive the lytic action of phage. Perhaps it should be emphasized that not all the phage-resistant mutants growing out after phage has acted on a strain are identical. Although similar in their resistance to phage the mutants obtained from a single strain may vary widely in other respects. For example, some of the phage-resistant mutants obtained by the action of phage on an avirulent strain were virulent whilst others remained avirulent but differed from the parent strain in their phage resistance and growth characters.

Reverting to the original working hypothesis that bacteriophage may be of significance in controlling bacterial variation in infections, the evidence so far collected indicates that, in the case of diphtheria, phages occur naturally, that they are able to effect rapid and significant changes in the characters of strains and that many strains encountered in infections present evidence of having been in contact with phage at some stage of their history. It is concluded, therefore, that the data available offer sufficient encouragement to continue the investigation.

REFERENCES

ANDERSON, J. S., HAPPOLD, F. C., McLEOD, J. W. & THOMSON, J. G. (1931). On the existence of two forms of diphtheria bacillus—*B. diphtheriae gravis* and *B. diphtheriae mitis*—and a new medium for their differentiation and for the bacteriological diagnosis of diphtheria. *J. Path. Bact.* **34**, 667.

BLAIR, J. E. (1924). A lytic principle (bacteriophage) for *Corynebacterium diphtheriae*. *J. infect. Dis.* **35**, 401.

BOYD, J. S. K. (1950). The symbiotic bacteriophages of *Salmonella typhi-murium*. *J. Path. Bact.* **62**, 501.

BRADSHAW, D. B., DIXON, C. W., MAWSON, F. M., TURNER, G. H. & ZINNEMANN, K. S. (1952). Diphtheria carrier investigation. *Lancet*, i, 558.

BURNET, F. M. (1930). Bacteriophage activity and the antigenic structure of bacteria. *J. Path. Bact.* **33**, 647.

CRAIGIE, J. & YEN, C. H. (1938). The demonstration of types of *B. typhosus* by means of preparations of Type II Vi phage. *Canad. publ. Hlth J.* **29**, 448.

EDWARD, D. G. FF. & ALLISON, V. D. (1951). Diphtheria in the immunized with observations on a diphtheria-like disease associated with non-toxigenic strains of *Corynebacterium diphtheriae*. *J. Hyg., Camb.* **49**, 205.

ELFORD, W. J. & ANDREWES, C. H. (1932). The sizes of different bacteriophages. *Brit. J. exp. Path.* **13**, 446.

FELIX, A. & ANDERSON, E. S. (1951). Bacteriophage, virulence and agglutination tests with a strain of *Salmonella typhi* of low virulence. *J. Hyg., Camb.* **49**, 349.

FISK, R. T. (1942). Studies on staphylococci. I. Occurrence of bacteriophage carriers among strains of *Staphylococcus aureus*. *J. infect. Dis.* **71**, 153.

FREEMAN, V. J. (1951). Studies on the virulence of bacteriophage infected strains of *Corynebacterium diphtheriae*. *J. Bact.* **61**, 675.

FREEMAN, V. J. & MORSE, I. U. (1952). Further observations on the change to virulence of bacteriophage-infected avirulent strains of *Corynebacterium diphtheriae*. *J. Bact.* **63**, 407.

GRATIA, A. (1936). Des relations numériques entre bactéries lysogènes et particulier de bactériophage. *C.R. Soc. Biol., Paris*, **122**, 812.

HEWITT, L. F. (1947). Serological typing of *C. diphtheriae*. *Brit. J. exp. Path.* **28**, 338.

HEWITT, L. F. (1948*a*). Virulence and toxigenicity of different serological types of *C. diphtheriae*. *Brit. J. exp. Path.* **29**, 181.

Hewitt, L. F. (1948*b*). Protection by streptomycin, penicillin and licheniformin against *C. diphtheriae* infections. *Brit. J. exp. Path.* **29**, 289.

Keogh, E. V., Simmons, R. T. & Anderson, E. (1938). Type-specific bacteriophages for *C. diphtheriae. J. Path. Bact.* **46**, 565.

Lwoff, A., Siminovitch, L. & Kjeldegaard, N. (1950). Induction de la production de bactériophages chez une bactérie lysogène. *Ann. Inst. Pasteur*, **79**, 815.

Parsons, E. I. & Frobisher, M. (1951). Effect of bacteriophage on virulence of *Corynebacterium diphtheriae. Proc. Soc. exp. Biol., N.Y.* **78**, 746.

Römer, P. H. (1909). Über den Nachweis sehr kleiner Mengen des Diphtheriegiftes. *Z. ImmunForsch.* **3**, 208.

Smith, G. H. & Jordan, E. F. (1931). *Bacillus diphtheriae* in its relationship to bacteriophage. *J. Bact.* **21**, 75.

Stone, F. M. & Hobby, G. L. (1934). A coccoid form of *C. diphtheriae* susceptible to bacteriophage. *J. Bact.* **27**, 403.

Toshach, S. (1950). Bacteriophages for *C. diphtheriae. Canad. J. publ. Hlth*, **41**, 332.

Wilson, G. S. & Atkinson, J. D. (1945). Typing of staphylococci by the bacteriophage method. *Lancet*, i, 647.

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REES, R. J. W. & HART, P. D'A. (1952). *J. gen. Microbiol.* 7, 372–381.

The Inhibition of Growth of Avirulent Strains of *Mycobacterium tuberculosis* by a Surface-active Polyoxyethylene Ether

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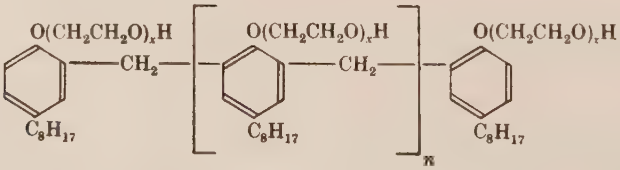
SUMMARY: Examination of the action of a series of surface-active polyoxyethylene ethers on the growth of the avirulent strain of *Mycobacterium tuberculosis*, H37Ra, showed that one member, the ether of 2, 2′-dihydroxy-5, 5′-di-*tert*-octyldiphenylmethane (D4), inhibited this strain but not the virulent variant H37Rv. This type of differential inhibition has been observed with a commercial surface-active mixture, 'Triton A20' (Dubos & Middlebrook, 1948). Lengthening of the polyoxyethylene chains of D4 led to loss of its inhibitory property. Both D4 and 'Triton A20' could induce resistance in H37Ra, and cross-resistance to D4 of a strain resistant to 'Triton A20' was observed; this cross-resistance suggested that a compound closely related to D4 was the active constituent of 'Triton A20'. The resistant strains grew in a microscopically amorphous pattern, like their parent strain.

The growth of four other avirulent strains of tubercle bacilli was inhibited, like that of H37Ra, by D4, while two slightly virulent strains grew freely in this detergent, like H37Rv. One factor common to the strains naturally insusceptible to D4, but absent from those inhibited, is the ability to produce some degree of microscopic 'cord' pattern, and this property may have some connexion with the cause of the differential inhibition by D4. Surface activity also probably plays a part.

The development of experimental tuberculosis in mice and guinea-pigs has been shown to be markedly suppressed by the injection of non-ionic surface-active polyoxyethylene ethers, having the general formula given below, (Cornforth, Hart, Rees & Stock, 1951; Hart, Long & Rees, 1952). To obtain these agents, the polymeric condensation products of formaldehyde with *p*-*tert*-octylphenol were made water-soluble by reaction with ethylene oxide, the resulting polyoxyethylene chains being of predetermined average length. For the preparation of the lower members of the series, with one, two or three phenolic nuclei (monocyclics, 'twins' or 'triplets'), the corresponding phenols were available in pure crystalline form, and could be separately condensed with ethylene oxide. The higher members (tetracyclic, etc.) have not so far



$n = 0, 1, 2, 3$, and higher integers. $x = 10, 20, 30$, or 60 (average value).

been individually prepared, their precursors being resinous mixtures of polycyclic phenols contaminated with the lower di- and tricyclic phenols.

Of our products in which the polyoxyethylene chains were of medium average length ($x=20$, see Formula), only the monocyclic compound (D3, see below) appreciably inhibited *in vitro* the growth of the virulent strain of *Mycobacterium tuberculosis* (H37Rv) used in previous chemotherapeutic experiments. This compound, however, was not antituberculous *in vivo*. On the other hand, both the 'twin' (D4) and the 'triplet' (D9*a*), which were chemotherapeutically inactive, as well as our chemotherapeutically active mixtures of compounds with larger numbers of phenolic nuclei (e.g. D2 and D2*a*), permitted growth at concentrations of up to 1·0 %.

We expected that the detergents would show similar *in vitro* inertness to avirulent strains of *M. tuberculosis*, but we were aware of the finding of Dubos & Middlebrook (1948) that at fairly low concentrations the commercial surface-active agent 'Triton A20' was inhibitory to the avirulent variant, H37Ra, but not to the virulent variant, H37Rv. This observation led us to look for a similar selective bacteriostatic property among members of the present series, whose chemical structure is similar to that of 'Triton A20', but which includes compounds derived from pure phenolic polymers. We were particularly interested in so doing because 'Triton A20' had toxic properties in the animal not shown by our comparable products, and it seemed possible that this agent's inhibitory power was due to some impurity.

MATERIALS AND METHODS

Surface-active agents. The chemical structure and average number of ethylene oxide units per phenolic nucleus (see Formula) are given in Table 1, molecular weights to the nearest 10 units being shown in brackets. 'Triton A20' was made by Rohm and Haas Co., Philadelphia, and supplied by Charles Lennig and Co., Ltd., London; it is stated to be a 25 % solution of an arylalkyl polyether of phenol. All the other products were synthesized by our

Table 1. *Details of surface-active agents used**

	Average number of ethylene oxide units per phenolic nucleus (x)							
Chemical structure	10		20		30		60	
	Serial no. (and mean mol. wt.) of detergent							
p-Octylphenol polyoxyethylene ether (monocyclics)	D15	(650)	D3	(1090)	D19	(1530)	D21	(2850)
$n=0$ ('twins')	D17	(1300)	D4	(2180)	D18	(3060)	—	—
$n=1$ ('triplets')	D16	(1960)	D9*a*	(3280)	D13	(4600)	—	—
$n=0, 1, 2, 3$, etc. (mixture)	D14†	—	D2	—	—	—	D22	—
As D2 but with less of lower polymers	—	—	D2*a*	—	—	—	—	—
'Triton A20'	—	—	—	—	—	—	—	—

* The products listed on any line are derived from the same phenolic precursor and differ only in the value of x (see Formula in text).

† If the mean molecular weight of D2 $=m$, that of D14 $=0{\cdot}61m$ and of D22 $=2{\cdot}6m$.

colleagues Dr J. W. Cornforth and Dr J. A. Stock. For bacteriostatic tests, 'Triton A20' 50 % (12·5 % effective agent), or our detergents in 6·25 or 12·5 % (w/v) solutions, were autoclaved, and were used at these strengths or at 1·0 %. Such solutions were stable for many months in the refrigerator. The stronger solutions were clear and amber-coloured; those of products with $x=10$ tended to be viscous. Solutions of all the agents at 1 % foamed on shaking. D14, D2, D2*a* and 'Triton A20' were chemotherapeutically active in experimental tuberculosis in the mouse.

Cultures. The strains of *M. tuberculosis* mainly used were H37Rv and H37Ra, virulent and avirulent variants of the human strain H37; these and four additional avirulent human strains, R1Ra, JH6Ra, JH16Ra and H4Ra, were obtained from Dr W. Steenken, Jr., Trudeau Laboratory, New York (see Report, 1946; Steenken & Gardner, 1946*a*, *b*; Yegian & Kurung, 1952). A strain of BCG was obtained from Dr Knud Tolderlund, State Serum Institute, Copenhagen, and the degraded strain VAL from Dr F. C. O. Valentine, The London Hospital. The VAL strain was derived from H37Rv after repeated subculture in Tween albumin medium; its virulence in mice was considerably less than that of its parent strain, though greater than that of BCG.

Media and tests. The strains of *M. tuberculosis* were maintained on Herrold's (1931) egg-yolk agar medium, containing 5 % glycerol, or on the surface of Proskauer & Beck's defined medium containing asparagine (see Baldwin, Petroff & Gardner, 1927). For bacteriostatic tests, well-grown cultures were ground in Griffith tubes to give aqueous suspensions equal in opacity (by eye) to a standard containing 0·2 mg. (moist wt.)/ml. The basal medium used was: Difco Casamino acids, 2·5 g.; asparagine, 0·3 g.; anhydrous Na_2HPO_4, 2·5 g.; KH_2PO_4, 1·0 g.; sodium citrate, 1·5 g.; $MgSO_4, 7H_2O$, 0·6 g.; glycerol, 25 ml.; distilled water to 1000 ml. It was distributed in 5 ml. quantities in 25 mm. diam. test-tubes and autoclaved, and serial twofold dilutions of the detergents were made, giving concentrations ranging from 2·0 to 0·0004 % (w/v). To each tube 0·25 ml. 5 % albumin (bovine plasma fraction V) sterilized by filtration, and the inoculum 0·2 ml. of a standard suspension (0·04 mg. moist weight of bacilli), were added, and the tubes were incubated at 37° for 2 weeks. The maximum concentration permitting visible growth, or the minimum inhibitory concentration (which was twice the former value), was taken as end-point (readings made after 3 weeks' incubation did not materially affect the conclusions).

RESULTS

Effect of the different detergents on the growth of the avirulent strain H37Ra

Bacteriostatic tests were made with the agents having a medium polyoxyethylene chain length ($x=20$), using the H37Ra strain and, for comparison, strain H37Rv. Table 2 shows that the monocyclic compound D3 inhibited both strains, the avirulent little more than the virulent. The 'twin' D4, however, provides a striking contrast, this compound markedly inhibiting growth of H37Ra but not that of H37Rv. The 'triplet' D9*a*, on the other hand, was inactive against both avirulent and virulent strains, and so were the mixtures D2 and D2*a* containing higher polymers. The inactivity of D2 indicates a masking effect, since it contains at least 12 % of D4 as contaminant. The effect of 'Triton A20' is seen to be similar to that of D4, but the contrast is distinctly less. It may be noted that the growth of both strains was submerged in the presence of all the detergents, but that it was granular in D3 and dispersed in the others.

Table 2. *Effect of the surface-active agents (medium length of polyoxyethylene chains) on the growth of the virulent strain H37Rv and the avirulent strain H37Ra of* M. tuberculosis

Final concentration in medium (%)	Surface-active agent: D3 (monocyclic)		D4 ('twin')		D9*a* ('triplet'), D2 and D2*a* (mixtures)		'Triton A20'*	
	Rv	Ra	Rv	Ra	Rv	Ra	Rv	Ra
0·5	0	0	++d	0	+++d	++d	+d	0
0·2	0	0	++d	0	+++d	++d	+d	0
0·1	0	0	++d	0	+++d	++d	++d	0
0·05	0	0	++d	0	+++d	++d	++d	0
0·025	0	0	++d	0	+++d	++d	++d	0
0·012	±g	0	++d	0	+++d	++d	+++d	tr. d
0·006	+g	tr. g	++d	0	+++d	++d	+++d	±d
0·003	++g	±g	++d	0	+++d	++d	+++d	+d
0·0015	++g	+g	++d	tr. gd	+++gd	++d	+++d	++d
0·0008	++g	+g	+++g	+g	+++g	++g	+++g	++d
0	++g	+g	+++g	++g	+++g	++g	+++g	++g

0, tr., ±, +, ++, +++ indicate degrees of growth; and g, d, gd, indicate granular, dispersed or partly dispersed type of growth, respectively.

* The concentrations of 'Triton A20' refer to the effective agent.

Effect of varying the length of the polyoxyethylene chains

The lipophilic character of the molecule of these surface-active agents is determined by the arylalkyl groups. The hydrophilic character depends upon the polyoxyethylene chains, and can be varied by increasing or decreasing the average length of the latter attached by polymerization to a particular crystalline or resinous phenolic material. With this in mind, the phenolic precursors of the medium chain-length products ($x=20$), D3, D4, D9*a* and D2, were taken, and ethylene oxide polymerized on to them so that in each case several products were obtained differing only in their (predetermined) lengths of polyoxyethylene chains ($x=10$, 20, 30 and/or 60) (see Table 1). In this way a range of surface-active agents was created for each of the monocyclic, 'twin' and 'triplet' compounds, and also for the mixture composed mainly of higher polymers. The effect upon the bacteriostatic properties of the members of these four series, when x was increased or decreased from an average of 20, may be seen in Table 3 and Fig. 1. To show the inhibitory end-points, percentage concentrations (Table 3) have been converted (Fig. 1) into molar concentrations for the monocyclics, 'twins' and 'triplets'. For the higher-polymers series an arbitrary molecular weight of m has been assigned to D2, so that the other two variations of the mixture can be related to D2 also on a molar basis (this is permissible since all three products were derived from the same resin precursor and differ only in the relative amount of polymerized ethylene oxide).

Table 3. *Bacteriostatic power shown against strains H37Rv and H37Ra by the surface-active agents having varying average lengths of polyoxyethylene chains*

Maximum concentration (%) of detergent permitting growth of tubercle bacilli, virulent (Rv) or avirulent (

x*	Mono-cyclics	Strain		'Twins'	Strain		'Triplets'	Strain		Higher-polymers series	Strain	
		Rv	Ra		Rv	Ra		Rv	Ra		Rv	R
10	D15	0·006	0·003	D17	0·5+	0·0004	D16	2·0+	0·2	D14	2·0+	0·05
20	D3	0·012	0·006	D4	0·5+	0·0015	D9*a*	2·0+	2·0+	D2	2·0+	2·0
30	D19	0·025	0·012	D18	0·5+	0·5+	D13	2·0+	0·5+	—	—	—
60	D21	0·5+	0·05	—	—	—	—	—	—	D22	2·0+	2·0

* Average number of ethylene oxide units/phenolic nucleus. The products listed in any column are derived fr the same phenolic precursor and differ only in the value of x. (See Formula and Table 1.)

A tendency for inhibitory power (whether measured in percentage or in molar concentrations) to decrease with increasing polyoxyethylene chain length (from $x=10$ to $x=30$ or 60) may be seen. For the virulent H37Rv this is appreciable only in the monocyclic series, where the activity shown by D3 ($x=20$) is no longer evident in D21 ($x=60$), the latter compound failing to inhibit even at the highest concentration tested; on the other hand none of the 'twins', 'triplets' or products of the higher-polymers series were active against this strain over the range of concentrations used. In the case of the avirulent H37Ra the association is evident to some degree in each of the series. The inhibitory power of the monocyclic compounds against this strain decreases regularly, though slightly, with increasing chain length (from $x=10$ to $x=60$), while in the 'triplets' and also in the higher-polymers mixture the reduction of this length from $x=20$ to $x=10$ is associated with the appearance of inhibition at the highest concentrations used. In the 'twin' series the effect of the association is that the outstanding inhibition of H37Ra by D4 ($x=20$), already noted, is slightly enhanced when the polyoxyethylene chains are shortened (D17, $x=10$), but is lost when they are lengthened to $x=30$ (D18).

The macroscopic appearances of the submerged growth of both strains, whether granular or dispersed (see Table 2), were unaffected by these alterations of chain length, with two exceptions: (1) partly dispersed growth was given by the Rv strain in the higher concentrations of D21 and D22 (the only products with $x=60$); and (2) the dispersion of the growth given by this strain in D14 ($x=10$) was unusually fine. Microscopically, the Rv strain produced amorphous clumps when grown in the higher concentrations of the short-chained products D17, D16 and D14, instead of the well-marked 'cord' formation produced in all the other detergents. The Ra strain grew amorphously in all products.

Surface activity as a factor in the inhibitory properties of the detergents

The surface tension of the members of each of the series shown in Fig. 1, measured at fixed molar concentrations selected in the range of the bacteriostatic tests, was found to decrease as polyoxyethylene chain length (x) increased (Cornforth, Hart, Rees & Stock, in preparation). The fact, already

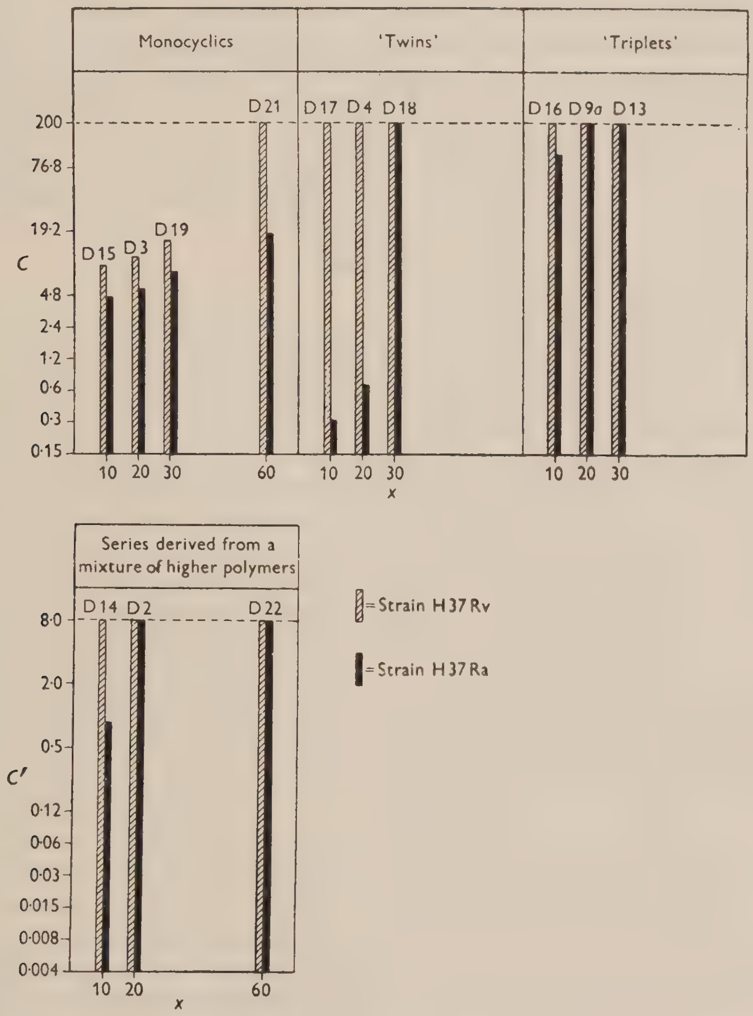


Fig. 1. Bacteriostatic power shown against *Mycobacterium tuberculosis*, strains H37Rv and H37Ra, by the surface-active agents having varying average lengths of polyoxyethylene chains. C = maximum molar concentration $\times 10^5$ of detergent permitting growth of tubercle bacilli. C' = maximum molar concentration $\times m$ (where m = mean mol. wt. of D2) of detergent permitting growth of tubercle bacilli. x = average number of ethylene oxide units per phenolic nucleus. Note: the horizontal interrupted lines at 200×10^{-5} M and at $\frac{8\cdot 0}{m}$ M indicate the highest concentrations charted; where C or C' is shown at one of these maxima, the actual value was, or may have been, even higher.

pointed out, that bacteriostatic power against the avirulent strain H37Ra also decreased as x increased suggests that the differences in this inhibitory power shown by the various products *within* each nuclear series (monocyclics, 'twins', etc.) is partly at least determined by differences in their surface activity. However, it is unlikely that surface activity is the main cause of differences between the bacteriostatic power of one nuclear series and that of another, particularly in the strikingly greater inhibition of the growth of H37Ra by 'twins' than by 'triplets'. Thus, at 25×10^{-5}M (*c.* 0·05 %) the surface tension of the medium-chained 'twin' D4 ($x = 20$) was 36 dynes/cm., while that of the short-chained 'triplet' D16 ($x = 10$) was 34 dynes/cm. Yet in spite of its higher surface tension, D4 inhibited the growth of H37Ra at 25×10^{-5}M and down to $0{\cdot}69 \times 10^{-5}$M (0·0015 %), whereas D16 permitted growth at 25×10^{-5}M and up to 102×10^{-5}M (0·20 %). A defect of these interpretations is, however, that surface tension indicates only surface activity at the air-liquid interface, and gives no direct information on surface forces at the cell-liquid interface.

Effect of the active 'twin' compounds on other attenuated and avirulent strains of M. tuberculosis

Each of the four avirulent strains R1Ra, JH6Ra, JH16Ra, and H4Ra was inhibited by the compound D4, much as was H37Ra. In a comparative assay the minimum inhibitory concentrations were, respectively, 0·0015, 0·006, 0·006 and 0·01 %, as compared with 0·003 %. As controls, the growth of H37Rv (inoculum 0·004 as well as the usual 0·04 mg.) was, as previously noted, not inhibited by D4 at 0·2 % (the highest concentration used), and none of the avirulent strains was inhibited by this concentration of D9*a* or of D2*a*. Neither BCG nor the VAL attenuated strain was inhibited to any marked extent by D4 or by D17, in the former case even when the inoculum was reduced to 0·004 mg. bacilli. Similar negative results were obtained with the saprophytic acid-fast strain ATCC607. The microscopic growth-pattern of these additional avirulent strains in the detergents was amorphous, like that of H37Ra. On the other hand, the BCG and VAL strains manifested some degree of cord formation, mixed with amorphous or semi-amorphous masses.

Production from H37Ra of strains resistant to certain of the detergents

Attempts to produce strains from H37Rv and H37Ra able to grow in concentrations of the monocyclic compound D3 greater than the latter's original minimum inhibitory values, using the conventional method of subculturing in increasing drug concentrations, were unsuccessful. However, strains capable of growing in high concentrations of the 'twin' D4 could be rapidly produced in this way from H37Ra. Thus, starting from the tube with the highest concentration of D4 (0·0015 %) permitting visible growth of H37Ra in an initial bacteriostatic assay, there emerged, after 7 serial transfers at 2–4 weeks intervals in increasing concentrations, a strain growing in as much as 2·0 % of this detergent. For an accurate final assay, suspensions prepared from egg-yolk agar cultures of this resistant strain and of the unmodified H37Ra were tested simultaneously for growth inhibition by D4, using comparable inocula: the inhibitory end-points were found to be >0.2 and 0·0015 %, respectively. In a similar manner, a strain was developed from H37Ra that would grow in high concentrations of 'Triton A20'. This strain was found to be also fully resistant to D4, but not to D3. The property of resistance to D4 and 'Triton A20' of these strains was stable after repeated subculture on egg-yolk agar.

Growth was well dispersed when the D4-resistant and the 'Triton A20' resistant cultures were grown in these two detergents, as was the case with the original sensitive H37Ra strain. Similarly, also, microscopic examination showed amorphous pattern of the bacilli; however, there was sometimes a tendency to parallelism of the organisms but no definite cord formation. Infection of mice with one of the strains resistant to 'Triton A20' showed no evidence of enhanced virulence.

DISCUSSION

We have confirmed the observation of Dubos & Middlebrook (1948) that the growth of the avirulent strain of tubercle bacillus H37Ra, but not that of the virulent H37Rv strain, is inhibited by the non-ionic surface-active agent 'Triton A20'. Extending this observation by means of the present agents, which form a series differing in the number of phenolic nuclei condensed and subdivided also according to the length of the polyoxyethylene chains, we have found the differential inhibition prominent only in the 'twin' series, but not in either of its neighbours, the monocyclic or the 'triplet' series. Monocyclics were inhibitory to the growth both of the virulent and of the avirulent strains, and this may be accounted for by the generally toxic character of the monocyclic series to biological systems (e.g. to other micro-organisms and to experimental animals). On the other hand, the 'triplets' and the mixtures composed mainly of higher polymers showed little or no activity against either strain. Among the active 'twins' we have paid particular attention to the compound D4, the polyoxyethylene ether of 2, 2′-dihydroxy-5, 5′-di-*tert*-octyldiphenylmethane, with an average of twenty polymerized ethylene oxide units per phenolic nucleus.

The connexion between our findings and the inhibitory action of 'Triton A20' on avirulent tubercle bacilli is not clear. The commercial agent's activity may be due to dicyclic compound, which it contains in significant amount, and in support of this is the cross-resistance to D4 of a 'Triton A20' resistant strain derived from H37Ra. However, our similar mixtures of higher polymers, such as D2, also contain 'twin' in about the same amount, but the latter's activity is masked therein. This apparent contradiction could be accounted for by other variables, such as differences in the length of polyoxyethylene chains of the components of 'Triton A20' and D2, respectively, or in preponderance of the various higher polymers.

The main effects of alterations in polyoxyethylene chain length on bacteriostatic activity against H37Ra are best considered with reference to the product in each nuclear series whose average number of ethylene oxide units per phenolic nucleus (x) is 20. When this medium chain-length compound inhibits H37Ra (as in the 'twin' and monocyclic series), this inhibition is increased when x is decreased below, and is diminished or lost when x is increased above, 20; of particular interest is the absence of activity of the 'twin' compound D18 which simply differs from the active D4 of the same series by having longer chains. When the medium chain-length compound of a series is inactive against H37Ra (as in the 'triplet' and higher polymers series), reduction of x to 10 is associated with the appearance of slight inhibitory power. Since changes in x are accompanied by changes in surface tension, these relationships suggest that surface activity, besides influencing dispersion of growth of all strains, contributes to the more specific phenomenon of inhibitory power against avirulent organisms. However, the fact that certain members of the 'twin' series are outstanding in their selective inhibition, but not in their surface tensions, indicates that surface activity is not the only factor responsible.

The bacteriostatic activity of these surface-active agents against avirulent organisms would not be expected to have any relationship to their antituberculous properties. Indeed, both the bacteriostatic D4 and the inert 'triplet' D9*a* are ineffective in experimental tuberculosis; and while the bacteriostatic 'Triton A20' is antituberculous, so also is the inert mixture D2. There is thus complete lack of correlation.

Although the 'twin' compound D4 differentiates avirulent tubercle bacilli (H37Ra and four other well recognized strains) from virulent bacilli (H37Rv) by the inhibition of the former strains, it does not distinguish between fully virulent and slightly virulent strains, for neither a culture of BCG nor our degraded strain of H37Rv (VAL) was inhibited. Indeed, on the basis of present experience, the growth of any strain of tubercle bacillus that shows some degree of 'cord' formation would seem to be unaffected by D4. Yet it is not just this microscopic growth pattern that decides the issue, one way or the other, because avirulent bacilli resistant to 'Triton A20' or to D4, obtained by exposure of H37Ra to increasing concentrations of detergent, still grew in a typical amorphous pattern.

Nevertheless, since the only strains of tubercle bacilli so far found to be inhibited by D4 have failed to manifest the slightest cord formation, we suggest as a possibility that the chemical constituents responsible for this growth pattern, and perhaps for virulence as well (see Bloch, 1950; Asselineau & Lederer, 1951) also protect organisms of lesser or greater virulence from the toxic action of D4. On this hypothesis: (1) the susceptibility of avirulent tubercle bacilli to D4 is attributable to the ability of this detergent to penetrate readily the cell membrane and then block or interfere with some metabolic process; (2) the production of resistant, but still avirulent, bacilli would be due to the use of some alternative metabolic pathway and (as we have shown) not by emergence of the ability to produce cords; (3) the native resistance of virulent tubercle bacilli to D4 is attributable to an imperviousness of their surface chemical structure (reflected in their growth pattern) to this detergent. This hypothesis is being tested by further experiments.

We are indebted to Dr J. W. Cornforth and Dr J. A. Stock for the series of surface-active agents, and to Dr W. Steenken Jr., Dr Knud Tolderlund and Dr F. C. O. Valentine for strains of *M. tuberculosis*.

REFERENCES

Asselineau, J. & Lederer, E. (1951). Recent researches on the chemistry of the lipids of the tubercle bacillus. *Experientia*, **7**, 281.

Baldwin, E. R., Petroff, S. A. & Gardner, L. U. (1927). *Tuberculosis: Bacteriology, Pathology and Laboratory Diagnosis*, p. 45. London: Baillière, Tindall and Cox.

Bloch, H. (1950). Studies on the virulence of tubercle bacilli. *J. exp. Med.* **91**, 197.

Cornforth, J. W., Hart, P. D'A., Rees, R. J. W. & Stock, J. A. (1951). Antituberculous effect of certain surface-active polyoxyethylene ethers in mice. *Nature, Lond.* **168**, 150.

Dubos, R. J. & Middlebrook, G. (1948). The effect of wetting agents on the growth of tubercle bacilli. *J. exp. Med.* **88**, 81.

Hart, P. D'A., Long, D. A. & Rees, R. J. W. (1952). Depression of tuberculin sensitivity in guinea-pigs by certain antituberculous surface-active agents. *Brit. med. J.* **1**, 680.

Herrold, R. D. (1931). Egg yolk agar medium for the growth of tubercle bacilli. *J. infect. Dis.* **48**, 236.

Report (1946). A depot for standard cultures of tubercle bacilli. *Amer. Rev. Tuberc.* **53**, 511.

Steenken Jr., W. & Gardner, L. U. (1946*a*). R1 strain of tubercle bacillus. *Amer. Rev. Tuberc.* **54**, 51.

Steenken Jr., W. & Gardner, L. U. (1946*b*). History of H37 strain of tubercle bacillus. *Amer. Rev. Tuberc.* **54**, 62.

Yegian, D. & Kurung, J. (1952). Growth-pattern and virulence of tubercle bacilli. *Amer. Rev. Tuberc.* **65**, 181.

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MAYR-HARTING, A. (1952). *J. gen. Microbiol.* 7, 382–396.

The Phage Typing of *Shigella sonnei*, and the Limits of Type Stability

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SUMMARY: 560 strains of *Shigella sonnei* were typed by Hammarström's method using bacteriophages, and the results tabulated by districts and outbreaks. Experimentally it was found that by the action of different phages transformation to a more resistant type could be achieved without conferring lysogenicity. The mechanism of this transformation seems to be in most cases the selection by the phage of true mutant types of which several are present in small numbers in every Sonne strain. The mutant types obtained most frequently out of type 3 cultures by experimental methods were also found in association with type 3 outbreaks. As dysentery phages are present in the intestines of many people, it is postulated that such transformations occur *in vivo* and that difference of type does not mean lack of recent genetic relationship.

Cooper & Mayr-Harting (1951), who made a preliminary survey of the epidemiology of Sonne dysentery in Bristol, by the phage-type method, stated that 'much remains to be done to establish the degree of stability of the types *in vivo* and *in vitro*'. The present investigation falls naturally into two parts: (1) an examination of type stability in the laboratory, and (2) a further investigation into the epidemiological significance of these types.

Phage-typing of certain organisms has become firmly established as a method of epidemiology, but the results are not easily checked because the method is used by only a few people. The results are usually accepted as an absolutely reliable answer to the inquiry for the pathways of an outbreak. It is, therefore, of some use to examine the development of this method and the technique, before trying to interpret our own typing results.

Phage-typing became a possibility after Burnet (1927) showed for the first time that sensitivity to certain antibodies was parallel to sensitivity to certain bacteriophages. Later Sonnenschein (1928) used specific typhoid and paratyphoid phages, not to replace the serological examination or to complement it, but to speed up the diagnosis, as a positive result could be obtained after 4 hr. Phages were soon used extensively in Germany (Schmidt, 1932*b*) for quick diagnosis, but they were not used to differentiate diverse types which could not be diagnosed by serological means.

Sertic & Boulgakov (1936*a*) described phages to which only typhoid strains containing the Vi antigen were sensitive, and later in the same year Scholtens (1936) and Craigie (1936) obtained results which led to the same conclusions.

Most phages investigated had their point of attack at a somatic antigen, but there are a few reports of phages which attack at flagellar antigens. Sertic & Boulgakov (1936*b*) described a phage lysing flagellated typhoid bacilli, inactive on non-motile variants. And Sievers (1943) reported the finding of the new salmonella type 'Köln' by its reaction to a phage which must have been flagellar. The strain in question gave the serological reactions of *Salmonella typhimurium* but was resistant to the *Salm. typhimurium* phage, subsequently it was found serologically to differ in its specific phase from *Salm. typhimurium.* The Breslau phage used by Schmidt (1932*a*) seems to have been a flagellar phage too. These phages of Schmidt's were 'adapted', i.e. he used Sonnenschein's paratyphoid phage and adapted it by numerous subcultures to other salmonella types as required.

It was observed by Craigie & Yen (1938) that a phage attacking the typhoid Vi antigen could be narrowed down in specificity to certain strains, and that this phage by a process of adaptation could be made to pick out specifically other strains. All strains picked out by one such adapted phage were assumed to be of one type. This adaptation was not an absolute one, i.e. only at certain dilutions of the phage could differences be observed.

However, a subdivision of typhoid strains could thus be achieved, and this method was put to practical use by Felix (1943). Felix & Callow (1943) extended the method to the typing of *Salm. paratyphi B*, adapting paratyphoid B phages in the same way.

Wilson & Atkinson (1945), in the phage typing of staphylococci, used seven phages derived from various strains of staphylococci; eleven further phages were developed by growing one of the original phages in the presence of a strain to which it was to be adapted 'in much the same way as with Craigie's typhoid phages'. The adaptation into typing phages each reacting with only one type of organism could not be completely achieved. Of their fourteen types, types 1, 2 and 3 are really groups of subtypes each reacting with one or more of a group of phages. Similarly, each phage of that group reacts with one or more subtypes within a type. These subtypes are recognizable by a certain pattern of phage sensitivity, and not by sensitivity to one phage.

Five other species for which phage typing has been studied consist each of only one group of subtypes. These are *Salm. typhimurium* (Lilleengen, 1948) the lactic streptococci (Nichols & Hoyle, 1949), Sonne's bacillus (Hammarström, 1949), *Salm. thompson* (Williams Smith, 1951*a*) and *Salm. dublin* (Williams Smith, 1951*b*).

Of these the phage typing of dysentery has been described in the greatest detail by Hammarström, who used twelve phages. His phage XII acts on all Sonne strains that are in the particular phase in which they are suitable for typing; Hammarström calls this the R phase, which seems to correspond with the phase II described by Wheeler & Mickle (1945). His eleven other phages differ qualitatively and quantitatively, so that a very large number of reaction patterns is possible. The quantitative differences make it necessary to use each phage in a 'critical test dilution'; only so can the characteristic pattern be obtained.

Hammarström has studied technique very carefully and gives accounts of the changes of reactions brought about by the minutest changes in the composition of the nutrient medium, the drying of the plates, etc. So far, nearly a hundred types have been isolated in several thousand typed strains.

METHODS

Organism. Rough variants are used for phage-typing of Sonne strains; fresh cultures straight from deoxycholate medium are not suitable. If subcultures on agar slopes are kept for a few days at room temperature they are found, on plating, to contain a large proportion of colonies showing the required rough morphology.

Phage. Owing to the fact that there is a critical phage concentration in which the phage must be used for the test, the phage preparations require a good deal of checking to detect slight losses of activity.

Usually it has been possible to prepare the test dilutions following Hammarström's table, but in a few cases the phages did not show the same relative activity on the standard strains; sometimes additional strains could be used to standardize the phage dilutions in these cases.

A difficulty which arose was that filtrates of strain 4 showed a marked inhibitory effect on the bacterial growth of a number of Sonne strains on agar. Eight of the twelve typing phages are propagated on strain 4 and these phage preparations, when used in fairly high concentrations, produced an inhibitory effect which could easily be mistaken for phage action where, in fact, the phage in the filtrate was inactive against the organism under test. When sufficiently diluted, this inhibitory substance ceased to affect growth at all, and did not give isolated plaques, as a phage would.

Filtrates of a broth culture prepared with the original freeze-dried strain 4 were much less inhibitory, and a new culture obtained from Dr Hammarström did not show inhibition at all.

Another irregularity was shown by phage XII, which normally produces large plaques, but when propagated with some cultures of strain 4, it throws off small-plaque variants at a rate of approximately 98 % and cannot be purified by repeated isolation from large holes. Whether these small-hole variants differ from the typical large ones in the activity of the phage-corpuscles concerned, has not been investigated.

Medium. At first 1½ % agar in broth prepared from Lemco 1 %, peptone Oxoid 1 % and NaCl 0·5 % with a pH of 7·6, was used. Later, on Dr Hammarström's advice, 1 % Difco Bacto-peptone was substituted for Oxoid peptone. Hammarström points out that the phage reactions vary a good deal with the medium, and undiluted phages showed a much greater activity on our medium than on Hammarström's. But as it was not my primary object to obtain typing results exactly comparable with Hammarström's, I retained the above medium which has the advantages of being cheap and available.

Typing. For typing, one colony is picked into nutrient broth and incubated overnight. Next morning 0·15 ml. broth culture is spread on an agar plate, the plate dried in the incubator for ½–1 hr., face up and lid removed, and then a small loopful of each of the twelve typing phages put on the plate in a definite order. The plates are read after 15 hr., not 5 hr. as in Hammarström's method.

Dr Hammarström kindly checked a large number of our typings and there were few discrepancies, but it is still possible that some of the strains which

I typed as type 3 may be, on Hammarström's medium, less sensitive and belong to another type. On the other hand, I found that strains which Hammarström typed as type 17, fell on our medium into two distinct groups with entirely different patterns of phage sensitivity. I have called these 17·1 and 17·2 respectively.

RESULTS

Experimental work on type stability

This was undertaken with the purpose of gaining some idea of the stability of types; and to see whether, if the types were not stable, a trend of development from certain types to certain other types could be ascertained.

(*a*) *Types of subcultures from individual colonies.* In a number of preliminary experiments agar slope cultures of organisms of various types were plated out and ten colonies picked into broth and typed. No variation was noticed in any of these experiments, but the agar slopes were subcultures of one colony and the variation rate, if type variation existed, would have to be very high to show up in experiments involving this small order of magnitude.

(*b*) *Types of different colonies from the same patient at one time.* In another experiment ten colonies were picked up from a direct culture of faeces on desoxycholate, but these colonies, as was to be expected, were not rough, and only one of them was typical type 3, the others showing varying degrees of resistance. From another direct culture in first isolation ten colonies were picked from desoxycholate into broth and subcultured after some days in fresh broth. These second subcultures gave typical rough flocculated growth and were all type 3.

(*c*) *Types of cultures from one patient at different times.* The strains from some patients who gave repeated positive cultures were typed at various times. There were, in all, seventeen cases in which repeat typings were carried out, eleven were type 3, one type 17, one type 19, one type 81, one type 82, one type 85 and one type 88. In no case was a change of type observed.

(*d*) *Effect on type of incubation with Hammarström's phages.* The results are given in Table 1. A typical type 3 strain, 5631, was inoculated into broth to which Hammarström's phage III had been added. After 4 days incubation the culture was plated out and nine colonies picked into separate broth tubes. Three of the colonies chosen were rough and six were smooth, as it was thought that amongst these latter the chance of finding a variant would be better. However, all three rough colonies and only one smooth one turned out to be type 79; the other five were slightly less sensitive and did not correspond to any known type (see Exp. 3). Strain 5631 was incubated subsequently with all Hammarström's phages and besides type 79 (see Exps. 4, 6 and 8), type 23 (Exps. 7 and 9) and type 66 (Exp. 4) were obtained from the same type 3 strain. These types were found to be stable after several weeks storage at room temperature and a number of subcultures. In addition, phage patterns occurred not corresponding to any type so far known. The phage concentration used for selection varied from 1/3 to 1/15 of the undiluted filtrates, but more dilute filtrates would be quite suitable as even 1/15 no longer yielded the

Table 1. *Patterns of phage sensitivity of cultures obtained by exposing strain* 5631 *type* 3 *to the action of Hammarström's type phages*

Exp.	Cultures obtained by growth in phage	Sensitivity pattern with type phages												Type obtained	No. of colonies tested
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII		
1	Control	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	3	
2	II	–	+	+++	+++	+++	–	+++	+++	+++	+++	+++	+++	Unknown	1*
3	III	–	+++	–	–	+++	–	+++	–	+++	+++	+++	+++	79	4
		–	+	–	–	+++	–	+++	–	+++	+++	+++	+++	Unknown	3
		–	–	–	–	+++	–	+++	–	+++	+++	+++	+++	Unknown	2
4	IV	–	+++	–	–	+++	–	+++	–	+++	+++	+++	+++	79	1
		–	+++	–	–	+++	–	–	–	–	+++	+++	+++	66	8
5	V	No viable growth obtained													
6	VI	–	+++	–	–	+++	–	+++	–	+++	+++	+++	+++	79	8
		–	+++	–	+	+++	–	+++	–	+++	+++	+++	+++	Unknown	2
7	VII	–	+++	+++	+++	+++	+++	–	+++	–	+++	+++	+++	23	4
8	VIII	–	+++	–	–	+++	–	+++	–	+++	+++	+++	+++	79	9
		–	–	–	–	+++	–	+++	–	+++	+++	+++	+++	Unknown	1
9	IX	–	+++	+++	+++	+++	+++	–	+++	–	+++	+++	+++	23	9
10	X	–	+	–	++	+++	–	+++	sc.	+++	–	+++	+++	Unknown	10
11	XI	–	+++	–	–	+++	–	+++	–	+++	+++	–	+++	Unknown	10
12	XII	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	Unknown	4
		Unreadable													3

+++ = confluent lysis; ++ = semi-confluent lysis; + = numerous separate plaques; sc. = scanty plaques; – = no lysis.

* Only one viable colony.

original type 3 strain and some of the more concentrated filtrates allowed only ghost colonies to grow in subculture, which on further subcultures were not viable.

(*e*) *Effect on type of incubation with patient's phages.* Transformation of types was then tried with phages isolated from the faeces of five children, suffering from Sonne dysentery.

The organisms isolated were referred to as A, B and C (type 3) and D and E (type 19). The corresponding filtrates from these faeces were called *a*, *b*, *c*, *d* and *e*. Two of the filtrates, *a* and *c*, were found to have slight activity against the type 19 strain E. All other cross tests were negative.

Further experiments with filtrates a and c. The phages present in filtrate *a* and *c* were each propagated by repeated incubation and filtration, with strain E and with strain 5631. This enhanced their activity considerably. The filtrates finally obtained were used, in the same way as the type phages previously used, to act on type 3 strain 5631 and also on type 19 strain E, and the changed phage sensitivities of the resulting growth were noted (Tables 2–4).

The following diagram will make clear how the filtrates were obtained and how the experiments, done with them, were connected:

Propagation by rapid subculture and filtration		Filtrate	Isolation of resistant organisms after:	Table
filtrate *a*	on strain E	W active only on strain E	further incubation with strain E	4
	on strain 5631	X active on strains 5631 and E	further incubation with strain 5631	2
			further incubation with strain E	3
filtrate *c*	on strain E	Y active only on strain E	further incubation with strain E	4
	on strain 5631	Z active on strains 5631 and E	further incubation with strain 5631	2
			further incubation with strain E	3

In both faeces more than one phage must have been present, as the phages propagated with strain 5631 attacked both this strain and strain E, whereas the phages propagated with strain E attacked strain E but not 5631. It is remarkable that the phages from the two faeces, both of which had been propagated with 5631 and which gave similar reactions with 5631 and E, must still be different, because the transformations which they caused in type 5631 gave different new types.

None of the patterns of phage sensitivity, obtained under the influence of phages from faeces, corresponded exactly to any known type; but three out of six colonies of strain 5631 grown in filtrate X gave a pattern which is at least very similar to type 79, and the same pattern was obtained under the influence of phage VI together with typical type 79. This pattern differs from type 19 only by its additional resistance to phage VIII, and phages VI and VIII are obviously closely related (Table 3).

Table 2. *Patterns of phage sensitivity of cultures obtained by exposing strain 5631, type 3, to the action of phages from patients' stools*

(Phages propagated on strain 5631)

	Strain	Patterns of phage sensitivity												Type	No. of colonies
Exp.	5631	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	obtained	tested
1	Grown in filtrate X	−	++	−	sc.	+++	−	+++	−	+++	+++	+++	+++	Unknown	2
		−	+	−	sc.	+++	−	+++	−	+++	+++	+++	+++	Unknown	1
		−	−	−	+	+++	−	+++	−	+++	+++	+++	+++	Unknown	3
2	Grown in filtrate Z	−	−	sc.	+	−	−	+	−	++	++	+++	+++	Unknown	2
		−	−	−	−	−	−	+	−	++	+	+++	+++	Unknown	2
		−	−	−	−	−	−	−	−	−	−	−	−	Resistant	2

+++ = confluent lysis; ++ = semi-confluent lysis; + = numerous separate plaques; sc. = scanty plaques; − = no lysis.

Tests for lysogenicity

Workers differ in their views regarding typing strains which have acquired resistance by becoming lysogenic. Williams Smith (1951*a*, *b*) denotes the type according to the pattern which they show in the normal phage-typing test. He even goes further and assumes that certain types only arise by acquiring lysogenicity, and thereby resistance, and for a species to be suitable for phage-typing a large proportion of its strains should be carriers of different phages. Nichols & Hoyle (1949) take the opposite view; when they find a lysogenic strain they classify it as if the strain were sensitive to the phage it carries, i.e. lysogenicity is taken as having the same significance for typing as sensitivity.

From a type 3 strain '8562', under the influence of phage IV and of phage VII, types 79, 66 and 23 were obtained. These were subcultured 6 times on Lemco agar, and then tested for lysogenicity by three different methods: (1) heating of broth cultures ½ hr. 56°; (2) filtration of broth cultures through Chamberland filters; (3) mixed culture with the sensitive original strain and subsequent filtration. The heated culture and the filtrates were then spotted on to a confluent inoculum of the original sensitive type 3, and another freshly isolated strain, also type 3. No phage action was found by any of these methods. The three variants were checked at this stage for phage-type and found to be type 79, 66 and 23 respectively. The significance of these experiments will be discussed later.

Table 3. *Patterns of phage sensitivity of cultures obtained by exposing strain E, type* 19 *to the action of phages from patients' stools*

(Phages propagated with strain 5631)

		Patterns of phage sensitivity													
Exp.	Strain E	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Type obtained	No. of colonies tested
1	Grown in filtrate X	–	++	+	++	+++	–	+++	–	+++	+++	+++	+++	Unknown	1
		–	++	–	sc.	+++	–	+++	–	+++	+++	+++	+++	Unknown	1
		–	+	–	–	+++	–	+++	–	+++	+++	+++	+++	Unknown	1
		–	–	–	+	+++	–	+++	–	+++	+++	+++	+++	Unknown	1
		Unreadable													2
2	Grown in filtrate Z	–	++	–	–	+++	–	+++	–	+++	+++	+++	+++	Unknown	1
		–	+	–	–	+++	–	–	–	+	++	+++	+++	Unknown	1
		–	sc.	–	–	+	–	–	–	+	++	+++	+++	Unknown	1
		–	sc.	–	–	sc.	–	–	–	sc.	sc.	+++	+++	Unknown	1
		–	–	–	–	sc.	–	–	–	sc.	sc.	+++	+++	Unknown	1
		–	–	–	–	sc.	–	–	–	–	+	+++	+++	Unknown	1

+++ = confluent lysis; ++ = semi-confluent lysis; + = numerous separate plaques; sc. = scanty plaques; – = no lysis.

Table 4. *Patterns of phage sensitivity of cultures obtained by exposing strain E, type* 19 *to the action of phages from patients' stools*

(Phages propagated with strain E)

		Patterns of phage sensitivity													
Exp.	Strain E	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Type obtained	No. of colonies tested
1	Grown in filtrate W	–	+++	–	–	+++	–	–	–	+++	+++	+++	+++	Unknown	1
		–	++	–	–	+++	–	–	–	+++	+++	+++	+++	Unknown	1
		–	–	–	–	–	–	–	–	+++	–	+++	+++	Unknown	1
		Unreadable													3
2	Grown in filtrate Y	–	–	–	–	sc.	–	++	–	++	sc.	+++	+++	Unknown	1
		–	–	–	–	–	–	++	–	++	+	+++	+++	Unknown	1
		–	–	–	–	–	–	++	–	++	–	+++	+++	Unknown	2
		–	–	–	–	–	–	sc.	–	++	–	+++	+++	Unknown	1
		Unreadable													1

+++ = confluent lysis; ++ = semi-confluent lysis; + = numerous separate plaques; sc. = scanty plaques; – = no lysis.

Typing results of Sonne dysentery from patients

The results of our typing are given in Table 5; 517 strains are from Bristol or the immediately adjoining districts, 43 are from other parts of the country; 455 (81·25 %) out of 560 strains were type 3. In Sweden, type 3 is the second most frequent with 23 %, and in Czechoslovakia, where Raška, Mališová & Mazáček (1950) did Sonne phage-typing, with Hammarström's phages, it is with 29·7 % the most frequent type. Where the number of strains investigated is sufficiently large, the distribution of types throughout Bristol city is not very different. A number of types occurred only once or twice. Of these, 12*a* was isolated from a boy who fell ill during his return journey from a French holiday; Dr Hammarström informed us that all his strains of 12*a*, without exception, came from France. We isolated three strains of type 66; two of these were isolated from patients, unrelated to each other, who had just been to France. The third strain of this type was obtained from a little girl who was found at the same time to carry *Salm. paratyphi* B, when at that time no other cases were known to exist in Bristol.

The predominant type in most outbreaks was type 3 and in a number of outbreaks this was the only type found.

Outbreaks of one type

Outbreak I. In a colony for mentally defective people thirty-six cases of type 3 occurred over a period of 1 year. No other type was found and it is assumed that all these infections were acquired at the colony.

Outbreaks II–IV involved twenty-two, seven and nineteen people respectively and were due to type 3.

Outbreaks VIII–X were small outbreaks in one hospital occurring over a period of 1 year.

Outbreak VIII was due to type 81. This type was for a short time found in a number of isolated cases. One of these was transferred to hospital and within a few days there was an outbreak in another ward involving about a dozen people. Nine of their cultures were typed as 81. No proof of the method of transfer has been obtained, but a member of the staff subsequently gave a history of intestinal upset. Strains of type 81 were not isolated after this outbreak.

Outbreak IX was only a very small outbreak. After the introduction of a case of type 3, three cases in the same ward, all close contacts, acquired the infection.

Outbreak X was rather involved, but it seems that it consisted of six cases of type 88 and that a few cases of type 21 and type 3 that were in hospital at the same time were independent of that outbreak and of one another.

Outbreaks of more than one type

Outbreak V. This involved fourteen people at a day nursery. Thirteen of the strains were type 3, one was type 85.

Outbreak VI and VII. These were large outbreaks at mental hospitals, one of fifty-five, the other of forty-one cases. The first yielded fifty-one cultures

Table 5. *Incidence of phage types of Sonne dysentery*

Place	Types of Sonne dysentery																					
	2	3	7	9	12*a*	17_1	17_2	19	21	23	28	32	36	50	66	79	81	82	85	87	88	
	No. of cases in area of outbreak																					
Bristol Central	.	32	.	.	.	.	.	.	2	1	.	.	.	.	.	1	1	.	2	.	.	39
Bristol East	.	81	.	.	.	.	1	.	7	2	1	.	.	1	1	.	1	.	2	.	.	97
Bristol West	.	6	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	7
Bristol North	1	36	.	.	1	1	.	.	6	4	.	.	.	.	.	1	2	.	4	1	2	59
Bristol South	.	42	.	.	.	.	.	.	1	1	.	.	.	.	.	.	.	2	3	.	5	54
Bristol surrounds	.	18	.	1	.	.	.	.	.	.	.	.	1	.	.	.	.	.	1	.	.	21
Warrington	.	12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	12
Leeds	.	7	1	.	.	.	.	.	.	1	.	3	.	.	.	.	.	.	.	.	.	12
Shenley	.	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
Canterbury	.	3	.	.	.	.	.	.	.	.	.	1	.	.	1	.	.	.	1	.	.	6
Folkestone	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
London	.	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4
Watford	.	4	.	.	?1	.	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	6
No. I outbreak	.	36	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	36
No. II ,,	.	22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	22
No. III ,,	.	7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	7
No. IV ,,	.	19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	19
No. V ,,	.	12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	.	13
No. VI ,,	.	51	.	.	.	.	.	1	.	.	.	.	.	.	.	2	.	.	1	.	.	55
No. VII ,,	.	38	.	1	.	.	.	.	.	2	.	.	.	.	.	.	.	.	.	.	.	41
Nos. VIII–X	.	13	.	.	.	.	.	.	.	.	.	.	.	.	.	.	9	.	.	.	6	28
Sporadic hospital cases	.	9	.	.	.	.	2	2	4	.	.	.	.	.	.	.	.	.	.	.	2	19
Total	1	455	1	2	2	1	3	3	20	12	1	4	1	1	3	4	13	2	15	1	15	560

of type 3, one patient had type 19, one type 85 and two type 79; from the other outbreak we obtained thirty-eight strains of type 3, two of type 23, one of type 9.

INTERPRETATION OF TYPING RESULTS AND GENERAL DISCUSSION

When Craigie & Yen first used their type classification of typhoid strains for epidemiological purposes they were well aware that it stood or fell with the stability of type. They did not obtain any experimental evidence of change of type in multiple isolations from one patient, nor in multiple colony isolations from the same culture, but they admitted that the natural transfer in man would offer greater opportunities. They say 'the isolation of different types of *B. typhosus* from apparently related cases would appear to demand further investigation since such a discrepancy would indicate that either the epidemiological information or the concept of type stability was erroneous'. In spite of this cautious dictum they were rather inclined to assume the stability of types. There has been a marked tendency lately to abandon even the caution and reserve that Craigie & Yen had expressed.

Table 6. *Phage sensitivity pattern of strains found in outbreaks together with type 3*

	Phage											
Type	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
3	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
85	–	+++	–	+++	+++	+	+++	+++	+++	+++	+++	+++
19	–	+++	–	+++	+++	–	+++	+++	+++	+++	+++	+++
79	–	+++	–	–	+++	–	+++	–	+++	+++	+++	+++
9	–	+++	+++	+++	+++	+++	–	+++	+++	+++	+++	+++
23	–	+++	+++	+++	+++	+++	–	+++	–	+++	+++	+++

+ + + = confluent lysis; + = numerous separate plaques; – = no plaques.

Felix (1943) states that the phage type of a strain is for all practical purposes a permanent character; a disquieting discrepancy is explained as an 'accident during the handling of the cultures'. Only in certain specified instances are changes of type admitted to occur (Craigie & Felix, 1947; Anderson, 1951).

Hammarström also has observed outbreaks in which a change of type occurred, and even changes of type in one patient. Some of these might indicate the introduction of a new strain but some he admits to be real changes of type.

In our material there are several cases where different types have occurred in seemingly related cases: (1) the outbreaks, V–VII, each with one or more cases of types other than the predominant type 3; (2) the very striking case of two brothers, one with type 3, the other with type 23; (3) two students in a training college, one type 3, and the other type 85. Only in the last case do the epidemiological data support the assumption that no genetical relationship exists between the two strains. In the outbreak V outside contacts existed so that the introduction of other types could not be excluded. In the other outbreaks, particularly in the example of the two brothers, a genetic relationship is likely although, owing to the widespread incidence of Sonne dysentery at the time, the introduction of other types cannot be excluded with certainty, nor is it certain that a mixed infection was not acquired, and different types survived in different people.

From the outbreak VI, two strains of type 79, one strain of type 85 and one of type 19 were obtained in an otherwise type 3 outbreak. In my experimental work I have shown that the transformation of type 3 to type 79 can be achieved by a number of different phages. Transformation of type 19, a type which also occurred in this outbreak, to a type closely resembling type 79 was actually achieved experimentally using a filtrate of a patient's faeces. Type 85 is only very slightly different from type 19. There is no doubt, therefore, that the four types that occurred in that outbreak have close genetic links and that the opportunity for the transformation existed *in vivo*.

In outbreak VII, two strains of type 23 and one of type 9 occurred; these are closely related to one another and type 3 is easily transformed into type 23 by the action of Hammarström's phages VII or IX. This transformation may also have taken place in the case of the two brothers with types 3 and 23.

In one experiment one phage, Hammarström's phage IV, acting on a type 3 culture yielded two different types, 79 and 66. It is reasonable therefore to assume some genetic relationship between these three types. Table 6 shows the phage sensitivity patterns of the strains concerned.

If the transformation of types by phages occurs in the field, any unusual type isolated may have one of two sources: (*a*) a carrier of that type; (*b*) a carrier of a bacteriophage which can transform a more common type into the one in question. One case reported by Felix (1943) can be explained on these lines. A new typhoid type D5 was isolated from an Indian soldier who fell ill several months after his arrival in this country. Felix interprets this as the strain having been imported from India, but it is possible that the man imported a particular bacteriophage, became infected with a D strain in this country, and the transformation to D5 was effected by the bacteriophage.

Similarly, the patients who fell ill with type 66 dysentery after their return from France may have picked up the strain there, or they may have picked up a phage, perhaps common in France, which transformed an ordinary type 3 strain into type 66.

In an epidemiological investigation phage typing of strains alone may be misleading. Where, in an outbreak, different phage types are encountered, only an analysis of the pathways through patients and carriers of the bacteriophages as well as those of the infecting strains will provide the true story; a truly formidable task.

Nicolle and his co-workers (1951*a*–*c*) found that the phage types of *Salm. paratyphi* B were largely determined by their lysogenic properties, and most naturally occurring types could be produced artificially by infecting an appropriate basic type with the appropriate Vi phage. The three basic types 1, 3*a* and 3*b*, could not, on the other hand, be transformed into one another by means of the Vi phages which they carried. Nicolle concludes, therefore, that lysogenicity is not the only factor that determines the phage type. He suggests that the lysogenic phage types may have arisen by contamination of the basic types with phages encountered in the intestine.

Williams Smith (1951*a*, *b*), using 0 phages for the typing of *Salm. thompson* and *Salm. dublin* had similar results. Some phage types were dependent on acquired resistance to a certain phage, others not. Some of his strains of *Salm. dublin*, made resistant to two phages, were found to carry only one. This leads to the conclusion that in that group of organisms even acquired phage resistance is not necessarily associated with lysogenicity.

The three variant types which I obtained from a type 3 strain were free from phage infection, and as the phage which they would have carried was known, it could not have been missed by using insensitive indicator strains. The process of change of type, in the case of Sonne dysentery, seems to be due to the presence of a number of true spontaneous mutants, far too small in numbers to show up in the usual typing test, but selected in a medium which contains a phage to which they are resistant. In support of this is the fact that the new types were resistant to other phages besides the one used for their selection, and that under the influence of one phage on one strain two types could be obtained, having in common resistance to the selecting phage, but otherwise differing in their pattern of sensitivity.

From the literature and from my own experimental results it seems that under the influence of a phage the phage type of an organism can be changed *in vitro* in two fundamentally different ways: (1) by acquiring lysogenicity and thereby resistance to that particular phage; (2) by the selection of pre-existent mutants resistant to the phage.

We do not know much about the role which these transformations play in the field. Williams Smith (1948) has found evidence of change of type of staphylococci in infected udders due to the influence of bacteriophage. Bacteriophages are known to be present in every intestine, and opportunities for the interaction of bacteria and phages are likely to occur.

The high incidence and ubiquitousness of Sonne dysentery might still allow one to argue the occurrence of unrelated strains of different type in one outbreak. In the writer's limited experience of typhoid and paratyphoid outbreaks there are two examples where multiple phage types were found when multiple infection seemed, on epidemiological considerations, extremely unlikely.

Vi-phage types may be more stable than 0-phage types in the field. Felix (1943) pointed out that, whereas typhoid 0-phages are very frequently found in stools, the finding of Vi phages is rather rare. This may be the reason for the stability of Vi types, as compared with Sonne types. But the Vi-type is not influenced by Vi phages only, as Anderson (1951) showed when he demonstrated that the change of type F1→F2 is dependent on an 0-phage.

In the evaluation of phage-typing it is usual to argue from the typing results, as *a priori* correct, to the epidemiological information, thereby assuming an absolute stability of phage type. This assumption seems to me open to grave doubt, and much useful knowledge might be gained by accepting instability *in vitro* as an established fact and instability *in vivo* at least as a working hypothesis. As all findings of different phage types have hitherto been interpreted as due to different sources of infection we have no information on the incidence of change of type *in vivo*. Only when this is known for every organism, together with the genetic relationship between types, shall we be able to evaluate phage-typing results in a meaningful way.

I wish to thank Prof. K. E. Cooper for his continued interest and for much useful advice; Dr E. Hammarström who kindly sent us strains and phages and checked the type of numerous strains; Dr W. A. Gillespie in Bristol, Dr S. Bobra in Warrington, Dr G. M. Williamson in Leeds, and Dr G. B. Forbes in Canterbury for Sonne strains; Dr J. Macrae, Dr N. S. Craig, Dr Harris Caplan and Mr J. E. Mountjoy for epidemiological data and Mr W. L. Brawn for help with the typing.

REFERENCES

Anderson, E. S. (1951). The significance of Vi-phage types F1 and F2 of *Salmonella typhi*. *J. Hyg., Camb.* **49**, 458.

Burnet, F. M. (1927). The relationship between heat-stable agglutinogens and sensitivity to bacteriophage in the salmonella group. *Brit. J. exp. Path.* 8, 121.

Cooper, K. E. & Mayr-Harting, A. (1951). Phage-typing and epidemiology of *Shigella dysenteriae* Sonne. *Brit. med. J.* ii, 271.

Craigie, J. (1936). A bacteriophage for the Vi-form of *Salmonella typhi*. *J. Bact.* **31**, 56.

Craigie, J. & Yen, C. H. (1938). The demonstration of types of *B. typhosus* by means of preparation of type II Vi phage 1. Principles and Technique. 2. The stability and epidemiological significance of Vi-form types of *B. typhosus*. *Canad. J. publ. Hlth*, **29**, 448.

Craigie, J. & Felix, A. (1947). Typing of typhoid bacilli with Vi bacteriophage. *Lancet*, i, 823.

Felix, A. (1943). Experiences with typing of typhoid bacilli by means of Vi bacteriophage. *Brit. med. J.* i, 435.

Felix, A. & Callow, B. R. (1943). Typing of paratyphoid B bacilli by means of Vi bacteriophage. *Brit. med. J.* ii, 127.

Hammarström, E. (1949). Phage-typing of *Shigella sonnei*. *Acta med. scand.* (Suppl.), no. 223.

Hamon, Y. & Nicolle, P. (1951*b*). Recherches sur les facteurs qui conditionnent l'appartenance des bacilles paratyphiques B aux différents types bacteriophagiques de Felix et Callow. 2. Obtention expérimentale, à partir des cultures non lysogènes, des types de *S. paratyphi* B par contamination avec les bacteriophages extraits des types lysogènes. *Ann. Inst. Pasteur*, **80**, 496.

Lilleengen, K. (1948). Typing of *Salmonella typhimurium* by means of bacteriophage. *Acta path. microbiol. scand.* (Suppl.), no. 77.

Nicolle, P., Hamon, Y. & Edlinger, E. (1951*a*). Recherches sur les facteurs qui conditionnent l'appartenance des bacilles paratyphiques B aux différents types bacteriophagiques de Felix et Callow. 1. La lysogénéité des différents types de *Salmonella paratyphi*/B. *Ann. Inst. Pasteur*, **80**, 479.

Nicolle, P. & Hamon, Y. (1951*b*). Recherches sur les facteurs qui conditionnent l'appartenance des bacilles paratyphiques B aux differents types bactériophagiques de Felix et Callow. 3. Nouvelles études sur les transformations de types par l'action des bacteriophages extraits des bacilles lysogènes. *Ann. Inst. Pasteur*, **81**, 614.

Nichols, A. A. & Hoyle, M. (1949). Bacteriophage in typing lactic streptococci. *J. Dairy Res.* **16**, 167.

Raška, K., Mališová, V., & Mazáček, M. (1950). Praktický Význam Fagotypisace v Epidemiologii střevnich Nákaz. *Čas. Lék. čes.* **89**, 835.

Schmidt, A. (1932*a*). Gewinnung spezifisch eingestellter Diagnostikphagen durch Umzüchtung. *Zbl. Bakt.* (1. Abt. Orig.), **123**, 202.

Schmidt, A. (1932*b*). Über die Verwendung des Bakteriophagen in der Typhus- und Paratyphusdiagnose. *Zbl. Bakt.* (1. Abt. Orig.), **123**, 207.

Scholtens, R. Th. (1936). The resistance developed against bacteriophage. *J. Hyg., Camb.* **36**, 452.

Sertic, V. & Boulgakov, A. N. (1936*a*). Sur la sensibilité des souches d'*Eberthella typhi* au bactériophage avec les caractères antigèniques. *C.R. Soc. Biol., Paris*, **122**, 35.

Sertic, V. & Boulgakov, N. A. (1936*b*). Bacteriophages specifiques pour des variétés bactériennes flagellées. *C.R. Soc. Biol., Paris*, **123**, 887.

Sievers, M. (1943). Ein neuer Salmonellatyp 'Köln' durch Phagendiagnostik ermittelt. *Zbl. Bakt.* (1. Abt. Orig.), **150**, 52.

Sonnenschein, C. (1928). Bakteriendiagnose mit Bakteriophagen. *Dtsch. med. Wschr.* **54**, 1034.

Wheeler, K. M. & Mickle, F. L. (1945). Antigens of *Shigella sonnei*. *J. Immunol.* **51**, 257.

Williams Smith, H. (1948). Investigations on the typing of staphylococci by means of bacteriophage. II. The significance of lysogenic strains in staphylococcal type designation. *J. Hyg., Camb.* **46**, 82.

Williams Smith, H. (1951*a*). The typing of *Salmonella thompson* by means of bacteriophage. *J. gen. Microbiol.* **5**, 472.

Williams Smith, H. (1951*b*). The typing of *Salmonella dublin* by means of bacteriophage. *J. gen. Microbiol.* **5**, 919.

Wilson, G. S. & Atkinson, J. D. (1945). Typing of staphylococci by the bacteriophage method. *Lancet*, i, 647.

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In vitro and *in vivo* Properties of Culture Filtrates of *Bacillus subtilis* with High Gelatinase Activity

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SUMMARY: Concentrated culture filtrates from *Bacillus subtilis*, with high gelatinase activity, disintegrated hide powder although unable to attack native collagen; they also haemolysed the red blood cells of a number of species. The concentrated filtrates were dermonecrotic in rabbits and lethal on intravenous injection into mice. There was no conclusive evidence that the factors responsible for any of these effects were antigenic. Although each of the *in vitro* and *in vivo* activities was destroyed by heat, the haemolytic activity was more resistant than the others. Fresh normal sera from many species inhibited each of the three properties shown *in vitro*. Tests with gelatinase showed that inhibition occurred rapidly and in multiple proportions. The inhibitory action was diminished by heat and storage and was distinct from the antitryptic property of serum.

It is well known that many species of the genus *Bacillus* have the property of liquefying gelatin, and Bertiau (1914) originally showed that the enzyme involved in this reaction was present in culture filtrates separated from the bacilli. The activities of the enzyme, apart from its ability to liquefy gelatin, have not been extensively studied, and little is known of its action on other substrates. It was therefore considered desirable, especially in view of the interesting observations made during the last few years on the proteolytic enzymes of the related genus *Clostridium*, to investigate the activities of the gelatinase of certain species of *Bacillus*. The initial work reported here was made with culture filtrates of *B. subtilis*.

PREPARATION AND CONCENTRATION OF CULTURE FILTRATES

The medium found to be most suitable for preparing filtrates of high gelatinase activity consisted of 1 % Evans peptone in normal saline adjusted to pH 7·8. The medium was distributed in 250 ml. amounts in 20 oz. medical flats and autoclaved at 10 lb. for 10 min. No improvement in gelatinase production was obtained with media prepared either from other brands of peptone or from nutrient broth, and neither gelatin nor glucose added to the medium enhanced the production of enzyme.

Ten authentic strains of *B. subtilis*, from the Wellcome Laboratories and the National Collection of Type Cultures, were tested and found to show considerable differences in their ability to produce gelatinase. One of the most active strains, NCTC 3610, was chosen and used throughout this work.

Each bottle of medium was inoculated with 1 ml. of an overnight culture and incubated at 37° in an almost horizontal position to expose the maximum surface to air. Preliminary titrations by the method described below showed that the gelatinase titre reached its maximum in 2–4 days; this time was subsequently chosen for separating the filtrates, which was done through a Seitz EK filter. The gelatinase in the filtrate was concentrated by the addition of 500 g. ammonium sulphate/l., which precipitated the enzyme without appreciable loss of potency. The precipitate was filtered on Whatman no. 15 filter-paper and dialysed overnight against running tap-water to remove ammonium sulphate. The residue was then centrifuged to remove particulate material and the clear supernatant fluid dried from the frozen state.

IN VITRO PROPERTIES OF CULTURE FILTRATES

Action on gelatin

Titration. A method of titrating gelatinase based on the Frazier (1926) technique was first investigated. Dilutions of culture filtrate were pipetted into Heatley cups fixed on to the surface of non-nutrient agar plates containing 0·5 % (w/v) gelatin. After overnight incubation at 37° the plates were flooded with acid $HgCl_2$ solution and the activity of the enzyme estimated from the highest dilution which inhibited around the cup a zone of precipitation. This method was not adopted as the errors involved were found to be considerable and exact end-points were difficult to read.

A more reliable method was devised in which the gelatinase activity of culture filtrates was estimated from their ability to liquefy gelatin solution under standard conditions. Dilutions of filtrate differing by 10–20 % were made in tubes 1·7 × 9 cm. and to 1 ml. of each dilution, 1 ml. of 10 % (w/v) gelatin solution was added. The gelatin solution was prepared by dissolving flake gelatin (Griffin and Tatlock) in saline, adjusting to pH 7·8 and adding phenol to 0·5 % (w/v) as preservative. A control tube of 2 ml. of 1·5 % gelatin in saline was included with each titration. The mixtures of filtrate and gelatin, together with the control, were incubated at 37° in a water-bath for 1 hr. with periodic shaking and then transferred to a bath of ice water. The times taken for the mixtures to set to a consistency such that they failed to flow when the tubes were tilted horizontally were determined and plotted against the filtrate dilutions. A typical curve is shown in Fig. 1. The setting time of the control tube was also measured; this was consistently between 5 and 9 min., a time which lay on the steep part of the titration curve and enabled end-points to be read most accurately. The end-point was obtained from the graph by reading off the dilution of filtrate which gave the same setting time as the control. In the example shown in Fig. 1 the titre of the filtrate was 1/54. It has been found convenient to express gelatinase activities in terms of the number of minimal gelatinase doses/ml. (m.g.d./ml.) which is the reciprocal of the titre and for the filtrate in Fig. 1 is 54 m.g.d./ml. The majority of concentrated culture filtrates which we have prepared had a gelatinase activity such that a 1 % solution of the dried material contained 200–500 m.g.d./ml.

Effect of time of incubation. In most cases titrations of gelatinase were done after 1 hr. incubation of enzyme and gelatin mixtures. The sensitivity of the reaction was, however, increased by longer periods of incubation, and Fig. 2

indicates that there was a linear relationship between time of incubation and gelatinase titre. When it was necessary to measure low enzyme activities, which were not detected after 1 hr. incubation, a 24 hr. period was used.

Effect of pH *value.* Gelatinase titres did not vary appreciably when the mixtures of enzyme and gelatin were incubated within the pH range 6–8, but outside this range the titres were lower. Routine tests were made with mixtures at pH 7·8. The effect of the pH value on the stability of gelatinase was also determined. Portions of filtrate containing 32 m.g.d./ml. were maintained at different pH values for 24 hr. at 10° and the residual activity was titrated after the pH had been readjusted to 7·8. It was found that little or no loss in activity occurred between pH 5·0 and 11·0, but that outside this range the loss in activity was rapid.

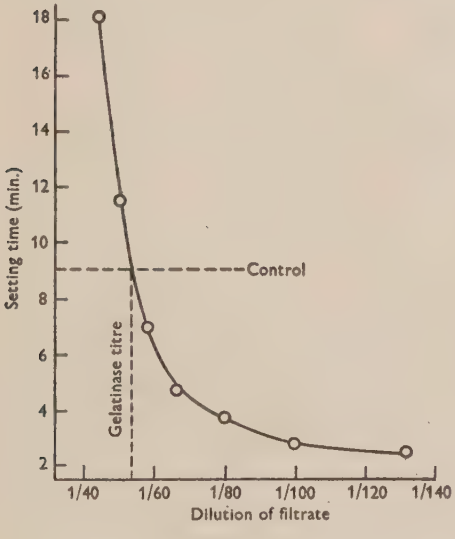

Fig. 1

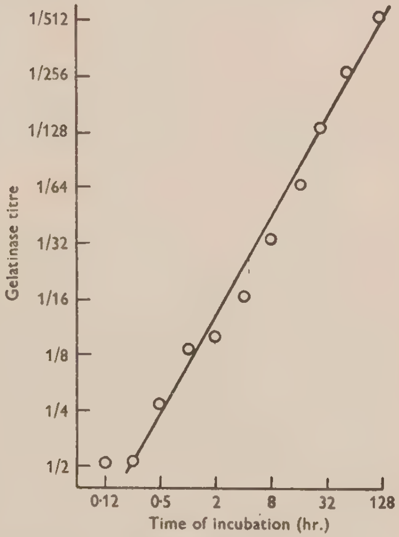

Fig. 2

Fig. 1. The relationship between setting time of gelatin and dilution of concentrated culture filtrate. The titre of this particular filtrate was 1/54 since this dilution gave the same setting time as the control tube.

Fig. 2. The effect on the gelatinase titre of the time of incubation of filtrate with gelatin. Logarithmic scales are used for both axes.

Effect of heat. This was investigated with a saline solution of filtrate containing 50 m.g.d./ml. No detectable activity was lost when the solution was kept at room temperature for 1 week, or at 37° for 24 hr. At higher temperatures, however, the enzyme was destroyed; in 2 hr. at 56° the activity was decreased by 50 % and at 65° inactivation was complete in less than 10 min. (Table 1).

Action on collagen and hide powder

Collagen. Solutions of dried culture filtrate containing 500 m.g.d./ml. of gelatinase were tested for their ability to disintegrate native collagen. Two collagen preparations were used: (*a*) thin shavings cut longitudinally from fresh ox Achilles tendon; (*b*) rat tail collagen 'paper' prepared by the method of Delaunay, Guillaumie & Delaunay (1949). These preparations were suspended in the enzyme solutions and incubated at 37°. There was no evident disintegration of either of these substrates after 48 hr. incubation. The inability of the culture filtrates to attack collagen was not attributed to the use of an insufficiently concentrated preparation, for it was found that a culture filtrate of *Clostridium histolyticum* containing only 16 m.g.d./ml. of gelatinase was able to dissolve with ease each of the collagen preparations which the *B. subtilis* filtrates containing 500 m.g.d./ml. had failed to attack.

Table 1. In vitro *and* in vivo *properties of a* 1 % (*w*/*v*) *solution of dried culture filtrate from* Bacillus subtilis *after exposure to various temperatures*

Temperature	Gelatinase (m.g.d.)	Hide powder enzyme (m.e.d.)	Haemolysin (m.h.d.)	Dermonecrotic factor (m.r.d.)
	Residual activity/ml. after 15 min.			
20°	240	640–1280	40	8
65°	<1	—	20	<1
70°	<1	<1	10	<1
80°	—	—	10	—
100°	—	—	<1	—

Hide powder. Although the culture filtrates were unable to disintegrate native collagen they attacked degraded collagen in the form of hide powder. This was shown by the ability of culture filtrates to produce zones of clearing by the hole-plate method using hide powder in agar plates (Evans, 1947) and to liberate dye from 'azocoll' (Oakley, Warrack & van Heyningen, 1946). The sensitivity of each of these methods was similar, and the activity of the hide powder enzyme was obtained by determining the minimal enzyme dose (m.e.d.) which produced a definite reaction. It was found that 1 % (w/v) solutions of dried culture filtrates contained about 500–1000 m.e.d./ml. The hide-powder disintegrating enzyme showed a sensitivity to the action of heat similar to that of gelatinase. A concentrated filtrate containing 600–1200 m.e.d./ml. lost its enzyme activity after it had been heated for 15 min. at 70° (Table 1).

Action on other substrates

Red blood cells. Culture filtrates were able to produce complete haemolysis of red blood cells. Three different species of R.B.C. were tested (rabbit, horse and guinea-pig) and were equally sensitive. Titrations of haemolytic activity were made in 0·15 M phosphate buffer saline at pH 6·9 by adding 1 ml. of a suspension of well-washed red blood cells to 1 ml. of each of a series of enzyme dilutions. The mixtures were incubated for 1 hr. at 37° in a water-bath and the minimal haemolytic dose (m.h.d.) determined from the highest dilution which produced complete haemolysis. Dried culture filtrates in 1 % solution were found to contain about 40 m.h.d./ml.

The haemolytic property of the concentrated filtrates was not as easily destroyed by heating as was the gelatinase activity. This is shown in Table 1 in which a comparison is made between the number of minimum reacting doses of gelatinase, hide powder enzyme and haemolysin in a filtrate which had been heated at various temperatures for 15 min. It is evident that whereas the gelatinase and hide powder enzymes were completely destroyed after 15 min. at 70°, the haemolytic activity was only decreased by 75 %. The Table also shows for comparison the effect of heat on the dermonecrotic property of the filtrate which is described in a later section.

Synovial fluid and lecithin. Filtrates were tested for hyaluronidase by the ACRA test (Burnet, 1948) and for lecithinase by the egg-yolk method (Macfarlane, Oakley & Anderson, 1941). There was no evidence that either of these enzymes was present.

INHIBITION OF *IN VITRO* REACTIONS BY NORMAL SERUM

Inhibition of gelatinase

Since attempts were to be made to prepare antisera capable of neutralizing gelatinase activity it was necessary first to investigate the known inhibitory action (Bertiau, 1914) of normal sera.

The inhibitory reaction. Twofold dilutions of serum were made in saline and to 1 ml. of each dilution, 1 ml. of filtrate containing 8–16 m.g.d. was added. The mixtures were held at 37° for 15 min. in a water-bath, and then 2 ml. of 10 % gelatin solution added to each mixture and incubation continued for a further 1 hr. Tests were read after the tubes had been cooled in the refrigerator and the highest dilution of serum which inhibited the liquefying action of the test dose of enzyme was determined. (In these titrations 1/1 means undiluted serum and <1/1 is used when undiluted serum showed no activity.)

Table 2. *Effect of time of incubation of serum and gelatinase, before addition of gelatin, on the titre of serum inhibitor*

Period of incubation at 37°...	0	5 min.	10 min.	30 min.	5 hr.
Titre of serum inhibitor	1/4	1/8	1/8	1/8	1/8

The inhibitory effect of fresh sera from seven different species (man, sheep, horse, ox, rabbit, guinea-pig and rat) was tested; with some of these species sera from several individuals were also tested. The inhibitory activities appeared to be a constant factor of normal serum, for in all tests the inhibitory titres were either 1/8 or 1/16. The inhibition of gelatinase by serum occurred in multiple proportions. This is shown in Fig. 3 by the linear relationship which was found to exist between the concentration of gelatinase and the dilution of serum which produced inhibition. The inhibitory action of serum was rapid. Tests in which gelatinase and serum were incubated for different times indicated that after 5 min. the reaction reached an equilibrium which did not change on further incubation for periods up to 5 hr. (Table 2).

Stability of the inhibitor. Titrations of gelatinase inhibitor were made with serum which had been heated at various temperatures. Table 3 gives the result

Table 3. *Effect of heat on the gelatinase inhibitory activity of normal serum*

Treatment of serum	Inhibitory titre
None	1/16
50° for 60 min.	1/16
60° for 30 min.	1/16
60° for 60 min.	1/8
70° for 5 min.	<1/1

of a typical test, and it is evident that the inhibitor was only slightly affected by 1 hr. at 60° but was rapidly destroyed at 70°.

The inhibitory action was also lost at lower temperatures although the rate of loss was not the same for all sera. Table 4 gives the inhibitory titres of a number of sera which had been stored at 5–10° for a few months, and it is evident by comparison with fresh sera that there had been considerable loss of inhibitory activity. The loss of inhibitory action was not due to the presence of 0·5 % phenol added to sera as preservative, for tests made with a serum sterilized by filtration and stored with and without phenol, showed that the decrease in inhibitory titres occurred at the same rate in each case. When sera

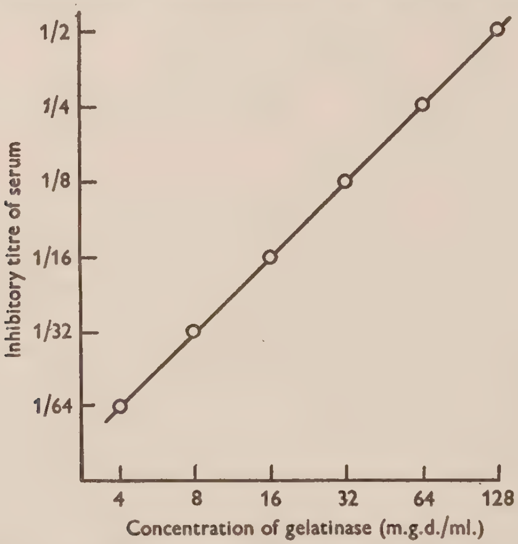

Fig. 3. The effect of various concentrations of gelatinase on the inhibitory titre of serum. Logarithmic scales are used for both axes.

Table 4. *Gelatinase inhibitory titres of fresh and stored normal rabbit sera*

	Titre
Twenty-one fresh sera	14 at 1/16 7 at 1/8
Twelve sera stored for 1–5 months at 5–10°	1 at 1/8 2 at 1/2 4 at 1/1 5 at <1/1

were kept at 37° the inhibitory effect was lost more rapidly. Sera from six rabbits each with an initial inhibitory titre of 1/8 showed after 12 days at 37° titres ranging from $<1/1$ to 1/2.

Comparison of gelatinase inhibitor with trypsin inhibitor. It is well known that serum has an antitryptic property, and the possible relationship of this property to the gelatinase inhibitor was investigated. This was done by comparing the trypsin and gelatinase inhibitory titres of fresh and stored sera. Typical results are given in Table 5, and it is evident from the varying ratios of the titres of gelatinase inhibitor to trypsin inhibitor that the factors responsible for these two effects were not the same.

Table 5. *A comparison of the titres of gelatinase inhibitor and trypsin inhibitor of normal sera stored for various periods*

Serum	Period of storage	Titre of trypsin inhibitory activity (A)	Titre of gelatinase inhibitory activity (B)	Ratio (A:B)
R9	1 day	1/6	1/12	2:1
R10	1 day	1/12	1/12	1:1
R63	1 week	1/6	1/12	2:1
R26	4 months	1/6	1/1	1:6
S1	9 months	1/12	1/6	1:2
H1	12 months	1/3	1/1	1:3

Inhibition of hide-powder enzyme and haemolysin

Preliminary experiments showed that the hide-powder disintegrating action and the haemolytic property of the filtrate were also inhibited by normal serum. Parallel titrations were made of the inhibitory action of a fresh normal rabbit serum using as indicators gelatin, azocoll and red blood cells. It was found that the inhibitory titres of the serum for gelatinase and hide-powder enzyme were identical at 1/14, while the haemolytic property was inhibited by serum diluted to 1/50. This result did not necessarily indicate that the haemolysin was distinct from the gelatinase and hide-powder enzyme since the filtrate contained a proportionately smaller number of minimal active doses of haemolysin, and therefore it might be expected that higher dilutions of serum would cause inhibition.

IN VIVO PROPERTIES OF CULTURE FILTRATES

Necrotizing property

The concentrated culture filtrates produced haemorrhagic necrotic lesions in the skin of rabbits (Pl. 1). A dose of 1 mg. contained in 0·2 ml. saline gave after 10–20 min. a flat area of oedematous induration 20–30 mm. in diameter. The centre of induration became red and then dark purple, and after 1–4 hr. the skin broke down and serous fluid exuded freely from the lesion. After 24 hr. the area was covered with a dark dry necrotic plaque 10–20 mm. in diameter. By injecting 0·2 ml. of a series of dilutions it was possible to titrate the necrotizing property of 1 % solutions of concentrated filtrates, taking as the minimal reacting dose (m.r.d.) the highest dilution which produced a definite necrotic spot. The m.r.d. of the most potent preparations was 0·25 mg. The filtrates when diluted well beyond the end-point of necrosis still produced induration.

The thermolability of the necrotizing factor was similar to that of gelatinase. A filtrate containing 8 m.r.d. was no longer active after it had been heated for 15 min. at 65° (Table 1).

The mode of action of the filtrates in producing skin necrosis has not been fully investigated, but the oedematous and haemorrhagic appearance of the lesions suggest that an increase in capillary permeability occurs at the site of injection. It was shown that the filtrates were, in fact, highly active in producing such an effect. This was done by titrating the filtrates intradermally in rabbits which had previously been given an intravenous injection of 4 ml. of a 5 % saline solution of Pontamine Sky Blue BX (Gurr). An intense blue staining rapidly developed at the site of injection, due to the leakage of dye-stained blood fluid into the cutaneous tissues. This effect was produced in less than 10 min. by a dose containing only 0·001 mg. of dry filtrate.

Lethal action

The lethal action of dried culture filtrate preparations was titrated intravenously in 20–25 g. mice. With the most potent preparations the minimum lethal dose (m.l.d.) was 2·5 mg. in 0·4 ml. saline and death occurred in 5–9 hr. Filtrates which had been heated for 15 min. at 65° were non-lethal when given in a dose of 5 mg. A fuller investigation of the lethal action cannot be made until more potent preparations are obtained.

ATTEMPTS TO PRODUCE ANTISERA TO CULTURE FILTRATES

Attempts were made to produce in rabbits, guinea-pigs and rats, antisera capable of neutralizing the gelatinase activity of culture filtrates. Three rabbits, eight guinea-pigs and eight rats were injected subcutaneously at weekly intervals for 6 weeks with concentrated culture filtrates containing 400–900 m.g.d./ml. of gelatinase. The total number of m.g.d. injected into each rabbit, guinea-pig and rat was, respectively, 17,000, 4000 and 4000. A second group containing two rabbits, eight guinea-pigs and eight rats was given a similar schedule of injections of whole living culture of *B. subtilis*. The cultures contained on the average 16–32 m.g.d./ml. of gelatinase, and each dose consisted of 10 ml. for the rabbits, 2 ml. for the guinea-pigs and 1 ml. for the rats. Samples of blood were taken from the animals 14 days after the last injection and the sera separated.

The sera were titrated for neutralizing action against gelatinase, using as controls sera from normal animals of the same species. The titration was made in the manner already described for estimating the inhibitory action of normal serum using a test dose of 1 ml. of filtrate containing 8–16 m.g.d. All the normal sera inhibited the gelatinase activity to the same titre of 1/10. The sera from all inoculated rabbits, however, had a titre to 1/20 which was shown by replicate tests to be a real difference. Similar differences were shown with sera from those guinea-pigs which received concentrated culture filtrate but not from those injected with whole culture. The sera from the inoculated rats all showed the same inhibitory titre as normal sera.

Those sera which showed titres higher than normal were titrated again after they had been held at 37° for a month—a treatment which, we had shown, diminished the normal inhibitory activity. Normal sera subjected to the same treatment served as controls. The titres are given in Table 6 together with those obtained for the sera before treatment. It is evident that incubation had the effect of decreasing the titres of all the sera, but the residual titres of the sera from the inoculated animals were substantially higher than those of the normal sera. This is suggestive but not conclusive evidence that the sera from the inoculated animals contained, in addition to the normal inhibitor of gelatinase, a neutralizing factor which may have been the result of the inoculation. Although the inoculated animals may have developed antibodies to gelatinase there was no evidence that they had developed a resistance to the necrotizing action of filtrates. Intradermal titrations of culture filtrates in the inoculated rabbits produced necrosis as rapidly and to the same titre as in normal controls.

Table 6. *Inhibitory titres of fresh and stored sera from normal and inoculated animals*

Preparation used for inoculation of animals	Animal	Gelatinase inhibitory titres of serum	
		Fresh	After 1 month at 37°
Concentrated filtrate	Guinea-pig no. 11	1/20	1/10
	Guinea-pig no. 12	1/20	1/10
	Guinea-pig no. 18	1/20	1/10
	Rabbit no. 3	1/20	1/5
	Rabbit no. 5	1/20	1/2·5
Culture	Rabbit no. 1	1/20	1/5
	Rabbit no. 2	1/20	1/5
None	Guinea-pig no. 19	1/10	1/1
	Guinea-pig no. 20	1/10	1/2·5
	Rabbit no. 6	1/10	1/1
	Rabbit no. 7	1/10	1/1

DISCUSSION

It has been known since the work of Bertiau (1914) that culture filtrates of *B. subtilis* possess the property of breaking-down gelatin, although little attention has been paid to their other properties. It is evident from our investigations, however, that there are a number of other definite activities associated with culture filtrates of this organism. So far only one strain has been used, but culture filtrates from this strain, with high gelatinase activity, contain an enzyme capable of breaking down degraded collagen in the form of hide powder and also a haemolysin active against the red blood cells of a number of different species. Further, the filtrates possess *in vivo* activities for they produce oedematous necrotic lesions on intradermal injection into rabbits and death on intravenous injection into mice. It is not yet known whether these *in vitro* and *in vivo* properties are associated with one and the same factor. A definite answer to this problem could be obtained if it were possible to produce neutralizing antisera, but so far we have no conclusive evidence that any of the factors is antigenic. We have, however, shown that the gelatinase, the hide-powder enzyme and the factors responsible for the *in vivo* reactions are similar in their thermolability, each being destroyed at 70° for 15 min., whereas the haemolysin appears to have a greater resistance to heat. This would at least suggest that the haemolysin is distinct from the other factors. On the other hand, the haemolysin appears to be similar to the gelatinase and hide-powder enzyme in that the activity of each of these three factors is inhibited by normal serum.

Although we have not yet been able to determine the relationship of the various *in vitro* and *in vivo* activities, it is interesting to speculate on the possible association of the 'proteolytic' properties with the production of dermonecrosis. The filtrates, although strongly active in breaking down degraded collagen in the form of gelatin and hide powder, were unable to affect native collagen and also unable to produce *in vitro* a softening effect on portions of skin. These observations suggest therefore that the disintegration and necrosis of skin following intradermal injection is not due to an enzyme in the filtrates which has a direct action on the collagen matrix of the skin, as is known to occur with the culture filtrates of *Cl. welchii*, type A (Oakley, Warrack & Warren, 1948) and *Cl. histolyticum* (Oakley & Warrack, 1950). Moreover, enzymes with the ability to break down gelatin and hide powder do not necessarily produce skin disintegration and necrosis since solutions of trypsin, 10 times more potent against gelatin and hide powder than *B. subtilis* culture filtrates, cause no necrotic reactions on intradermal injection into rabbits. It may be argued, however, that the inability of trypsin to produce necrosis is due to the fact that the enzyme is inhibited by serum, but such an argument does not apply as serum exerted a similar degree of inhibition against the 'proteolytic' action of the culture filtrates. It is therefore more probable that the development of skin necrosis was an indirect result of some other process occurring in the tissues. The filtrates were strongly active in producing a rapid increase in capillary permeability; doses containing less than 0·001 mg. of dry filtrate gave intense reactions within 5–10 min. after intradermal injection into rabbits containing circulating indicator dye. This property of the filtrate may be of primary importance, and be responsible indirectly for the production of dermonecrosis, but whether it is associated with the 'proteolytic' activity of the filtrates remains to be investigated.

The ability of fresh normal serum to inhibit the *in vitro* activities of culture filtrates has presented some interesting problems. Inhibition occurred with each of the three *in vitro* activities which we have examined, although our investigations were mainly confined to the action on gelatinase. The possibility was considered that inhibition might be due to a neutralization by naturally occurring antibody, but the constancy of the inhibitory titres of sera from different individuals and species is scarcely in support of this view. There was also the possibility that inhibition was caused by the presence in serum of a lytic enzyme which destroyed gelatinase. Our experiments do not support this, since inhibition was complete in less than 10 min. and did not progressively increase as would be expected of an enzyme reaction. A possible relationship of the gelatinase inhibitor to the trypsin inhibitor of serum was suggested by the fact that the inhibitory concentration of serum was directly proportional to the concentration of enzyme. This type of relationship was found for the trypsin inhibitor by Hussey & Northrop (1923) who suggested a combination of molecules of trypsin and inhibitor in definite proportions to form a 'trypsin-inhibitor complex'. We found the trypsin and gelatinase inhibitors to be distinct, for it was possible by storage of sera substantially to reduce the gelatinase inhibitor without diminishing the antitryptic power.

So far we lack conclusive evidence that the gelatinase of *B. subtilis* is antigenic; sera from animals inoculated with large doses of concentrated filtrate or whole culture were only slightly more active in inhibiting gelatinase than normal sera. They gave strong precipitin reactions with the gelatinase preparations, but since these preparations were far from pure it would be difficult to attribute this reaction to gelatinase. The inability of the enzyme to produce antibody may be associated with its susceptibility to the inhibitory action of serum. Such an action may involve a combination of enzyme with serum proteins and if it occurred *in vivo*, would have the effect of converting the inoculated enzyme from a heterologous to a homologous substance. With this hypothesis in mind an attempt was made to maintain the gelatinase in a heterologous form by mixing it with sheep serum in sufficient quantity to inhibit its *in vitro* activity. Sera from rabbits inoculated with this mixture were, however, no more capable of neutralizing gelatinase than normal sera, although they contained precipitins in high titre for sheep serum. Antibodies to other bacterial gelatinases have been obtained which are inhibited by normal serum such as the λ-antigen of *Cl. welchii* type B (Oakley *et al.* 1948); thus the lack of antigenicity shown by *B. subtilis* gelatinase need not necessarily be attributed to its inhibition by serum. The possibility of preparing antibodies to gelatinase by using adjuvants was also considered in view of the recent work by Duthie & Lorenz (1952) who prepared antibodies to staphylococcal coagulase by inoculating rabbits with the enzyme adsorbed on to $AlPO_4$. Our attempts, however, to adsorb gelatinase on to $AlPO_4$ or $Al(OH)_3$ were unsuccessful.

It is considered that the type of investigation described in this communication will be of value in the classification of species in the genus *Bacillus*. Other strains and species of the group may show characteristic differences in the *in vitro* and *in vivo* properties of their culture filtrates. We have already obtained evidence that the activities shown by *B. subtilis* filtrates *in vitro* on various collagen products are not completely reflected by some other actively 'proteolytic' species.

We should like to express our thanks to Mr H. Proom for strains of *B. subtilis* and to Dr G. H. Warrack for supplies of 'azocoll' and rat collagen paper. We are indebted to the Department of Medical Photography of the Manchester Royal

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REFERENCES

Bertiau, P. (1914). Les ferments bactériens qui liquéfient la gélatine et leur antiferments. *Zbl. Bakt.* (1. Abt. Orig.), **74**, 374.

Burnet, F. M. (1948). The mucinase of *V. cholerae*. *Aust. J. exp. Biol. med. Sci.* **26**, 71.

Delaunay, M., Guillaumie, M. & Delaunay, A. (1949). Études sur le collagène. 1. A propos des collagènases bactériennes. *Ann. Inst. Pasteur*, **76**, 16.

Duthie, E. S. & Lorenz, L. (1952). Staphylococcal coagulase; mode of action and antigenicity. *J. gen. Microbiol.* **6**, 95.

Evans, D. G. (1947). The production by certain species of *Clostridium* of enzymes disintegrating hide powder. *J. gen. Microbiol.* **1**, 378.

Frazier, W. C. (1926). A method for the detection of changes in gelatin due to bacteria. *J. infect. Dis.* **39**, 302.

Hussey, R. G. & Northrop, J. H. (1923). A study of the equilibrium between the so called 'antitrypsin' of the blood and trypsin. *J. gen. Physiol.* **5**, 335.

Macfarlane, R. G., Oakley, C. L. & Anderson, C. G. (1941). Haemolysis and the production of opalescence in serum and lecitho-vitellin by the α-toxin of *Clostridium welchii*. *J. Path. Bact.* **52**, 99.

Oakley, C. L. & Warrack, G. H. (1950). The alpha, beta and gamma antigens of *Clostridium histolyticum* (Weinberg & Séguin, 1916). *J. gen. Microbiol.* **4**, 365.

Oakley, C. L., Warrack, G. H. & Heyningen, W. E. van (1946). The collagenase (κ-toxin) of *Cl. welchii* type A. *J. Path. Bact.* **58**, 229.

Oakley, C. L., Warrack, G. H. & Warren, M. E. (1948). The kappa and lamda antigens of *Clostridium welchii*. *J. Path. Bact.* **60**, 495.

EXPLANATION OF PLATE

Development of oedema and necrosis in the skin of a rabbit after four successive intradermal injections of 0·2 ml. of 1 % solution of concentrated culture filtrate from *B. subtilis*. From left to right the injections were given 10 min., 1 hr., 4 hr. and 24 hr. before the photograph was taken. × ½.

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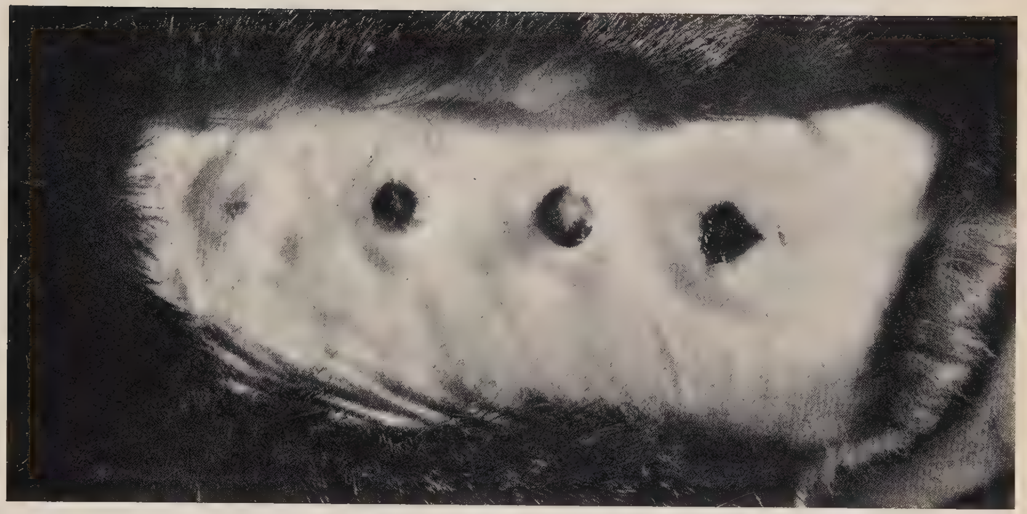

D. G. Evans & A. C. Wardlaw—Properties of *B. subtilis* culture filtrates. Plate 1

NEYLAND, M., DUNKEL, P. & SCHADE, A. L. (1952). *J. gen. Microbiol.* 7, 409–416.

The Uptake of Cobalt by *Proteus vulgaris*

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SUMMARY: *Proteus vulgaris*, suspended in meat extract broth or in a defined medium, bound cobalt to an extent dependent on the cell concentration and on the concentration of cobalt in the medium. An increase in concentration of cobalt or a decrease in the number of cells per unit volume resulted in a greater amount of cobalt ion bound per unit of cellular material. The composition of the suspending medium with regard to the cobalt combining groups present, as well as the availability of other metallic ions, markedly affected the amount of cobalt bound by the cells. Magnesium was particularly effective in diminishing the cobalt-combining capacity of the cells. An exposure time of 15 min. was sufficient for the major portion of the ion to be taken up by the cells. In an anaerobic medium, the cells bound approximately one-half as much cobalt as in an aerobic medium. Raising the pH value of the medium from 5·0 to 7·0 resulted in an increased uptake of the metal by the cells. Cobalt taken up by the cells could be removed to an extent of 70–80 % by dilution in distilled water and almost completely by 0·1 N-HCl. A derived strain of Proteus, capable of growth in relatively high concentrations of cobalt, bound smaller quantities of the ion at each level tested than did the parent strain. However, at the growth inhibitory level peculiar to each strain, the resistant form took up a much greater quantity of the metal than did the sensitive strain. This fact indicates that resistance to cobalt cannot be explained merely on the basis of the cobalt-concentrating ability of the cells.

The growth inhibitive action of cobalt on various bacteria and especially on *Proteus vulgaris* has been studied as a function of the composition of the growth medium, strain sensitivity, age of culture, size of inoculum, and other cultural conditions (Schade, 1949). Further, the effect of cobalt on the relationship between nucleic acid concentration and growth rate in the nucleic acids of *P. vulgaris* have been determined (Levy, Skutch & Schade, 1949*a*, *b*). Employing radio-isotope techniques, we have now investigated the uptake of cobalt by the cells of *P. vulgaris* under various environmental conditions in an effort to determine the principal factors involved in the binding of the metal.

METHODS

The organism employed in these studies was a strain of *P. vulgaris* isolated originally from a case of cystitis and maintained on meat extract agar slopes of the composition: 0·3 % (w/v) Difco meat extract; 1·0 % (w/v) Difco peptone; 0·5 % (w/v) NaCl; 3·0 % (w/v) Difco agar; adjusted to pH 7·5 with NaOH.

Cells from 18 to 24 hr. slope cultures were washed with either distilled water or 0·001 M potassium phosphate buffer, pH 7·3. The cell suspensions, made up to the desired turbidity, were then inoculated into the test medium containing cobalt as $CoSO_4.7H_2O$ and 0·03–0·07 μc./ml. of radio-active cobalt 60 as

$CoCl_2$. Following incubation in a water-bath at 37°, the radio-active cell suspensions were spun down in a Sorvall high-speed centrifuge in weighed 15 ml. glass centrifuge tubes. The supernatants were poured off and saved for determination of radio-activity. The tubes were then drained and weighed. From the known wet weight of cells calculated from the turbidity measurement, the amount of supernatant remaining in the tubes could be determined by difference. The cells were then suspended in 1·0 ml. 0·1 N-HCl and determinations of radio-activity made on 0·2 ml. portions of the acid cell suspensions and of their original supernatants. The samples to be assayed were pipetted on to glass cover-slips previously moistened with a drop of detergent (1 % Duponol, Dupont) to ensure even distribution of the radio-active sample. The total quantity of liquid was contained within a ring of Dow Corning heavy silicone stopcock grease. Samples thus prepared were dried under an infra-red lamp and counted with a Geiger-Müller counter.

RESULTS

Cobalt uptake as affected by the concentration of cobalt and cells in the medium

In previous studies of the inhibitive action of cobalt on the growth of *P. vulgaris*, we found that the extent of inhibition was conditioned by the concentration of cobalt in the medium and by the size of the inoculum. In a defined medium, 0·12 μg. Co^{++}/ml. was sufficient to prevent the growth of approximately 1000 cells/ml. At the same level of cobalt concentration, cultures containing 10,000 cells/ml. showed only partial inhibition and those containing 100,000 cells/ml. grew out to a degree equal to control cultures without cobalt.

To determine to what extent the amount of cobalt taken up by *P. vulgaris* was affected by the concentration of cobalt and the number of cells in a given volume of medium, we performed a number of experiments in which both of these factors were varied. In one set of experiments, a given weight of cells was exposed to increasing quantities of cobalt contained in equal volumes of 1:5 meat extract broth (MEB). Under these conditions, a fair degree of correlation was found between concentration of cobalt in the medium and uptake of the metal by the cells. The results of a typical experiment are summarized in Table 1. From these data, it is evident that an increase in the level of cobalt from 1 to 10 μg./ml. resulted in close to a tenfold increase in the amount of cobalt bound per mg. dry weight of cells. At the higher concentra-

Table 1. *Uptake of cobalt as affected by concentration of* Co^{++} *in the medium*

One mg. dry wt. cells suspended in 30·0 ml. of 1:5 MEB, pH 7·3 containing 1·0 μc. radioactive $^{60}Co^{++}$ and varying quantities of $CoSO_4.7H_2O$ to give the required concentration. Time of exposure 1 hr. at 37°.

Co^{++} concentration (μg./ml.)	Dry wt. of cells (mg./ml.)	Co^{++} uptake (μg. Co^{++}/mg. dry wt. cells)
1	0·033	0·237
10	0·033	2·12
100	0·033	15·3

tion of 100 μg. Co^{++}/ml., the increased uptake by the cells was no longer directly proportional.

When the concentration of cobalt was maintained at a constant level and the number of cells altered by the addition of varying dilutions of a stock-cell suspension, an inverse relationship was found between the amount of cobalt taken up per unit weight of cells and the concentration of cells in the medium. The results of two of such experiments are given in Table 2. The ability of the cells to concentrate high amounts of cobalt per unit of cellular material is particularly evident in those instances where the total cell number was low. The capacity of relatively low concentrations of cells to take up large quantities of cobalt was found also in experiments where variation in cell concentration was achieved in a different manner. In such tests, 0·5 mg. dry wt. of cells exposed to 100 μg. Co^{++}/ml. in 6·0 ml. 1:5 MEB took up approximately 19 μg. Co^{++}/mg. dry wt. cells, while the same weight of cells suspended in 30 ml. of this medium containing 100 μg. Co^{++}/ml. took up as much as 68 μg. Co^{++}/mg. dry wt. cells.

Table 2. *Uptake of cobalt as affected by concentration of cells in the medium*

Varying dilutions of a stock cell suspension were inoculated into 35 ml. of 1:5 MEB, pH 7·3 containing 1·4 μc. radio-active cobalt ^{60}Co^{++} in a final concentration of 100 μg. Co^{++}/ml. Time of exposure 1 hr. at 37.

Cell conc.	Total dry wt. of cells (mg.)	Co^{++} uptake (μg. Co^{++}/mg. dry wt. cells)
(*a*) ×1	0·093	86·4
×5	0·464	10·0
×24	2·24	4·92
(*b*) ×1	0·130	51·5
×4·7	0·612	8·81
×23·3	3·024	5·14

It can be noted here that in comparing different groups of inocula the actual amount of cobalt bound/unit of cell material varied, even though inocula of comparable age and size were exposed to the same concentration of cobalt. This variability of different inocula in their response to cobalt makes it difficult to obtain quantitative data necessary for elucidating the method whereby cell concentration affects the amount of cobalt bound.

Consideration of the data of Tables 1 and 2 led us to anticipate that if the concentrations of both cobalt and cells were simultaneously increased to the same degree, the observed uptake of cobalt by the cells would be the resultant of these two opposing factors. Such an experiment was set up by exposing equal quantities of cells to a given weight of cobalt contained in different volumes of suspending medium. The results summarized in Table 3 show that a decrease in the amount of cobalt bound/unit of cell material occurs as the concentration of both cobalt and cells is diminished. The amount of cobalt associated with the cells will be affected to a greater extent by a change in the concentration of cobalt in the medium than by an equivalent change in the concentration of cells.

Table 3. *Uptake of cobalt varying both* Co^{++} *and cell concentration*

2·0, 8·0, 32·0 ml. 0·9 % NaCl in 0·01 M sodium acetate, pH 6·0, containing: (*a*) 32 μg. cobalt, 0·5 μc. radio-active cobalt, 0·196 mg. dry wt./cells; (*b*) 2 μg. cobalt, 1·0 μc. radio-active cobalt, 0·223 mg. dry wt. cells.

	Co^{++} concentration (μg./ml.)	Cell concentration (μg. dry wt./ml.)	Co^{++} uptake (μg. Co^{++}/mg. dry wt. cells)
(*a*)	16	98	0·810
	4	24·5	0·369
	1	6·1	0·298
(*b*)	1	112	0·102
	0·25	27·9	0·0349
	0·0616	6·97	0·0171

Cobalt uptake as affected by the constituents of the suspending medium

Early in our work we had observed that the amount of cobalt taken up by the cells exposed to 10 μg. Co^{++}/ml. in a growth medium (1:5 MEB) was approximately one-third that taken up by an equal weight of cells in 0·001 M potassium phosphate buffer. The possibility that the metabolic state of the cells in a growth medium compared to that of cells in phosphate buffer might be reflected in their cobalt-combining capacity was investigated. Since, however, the MEB has cobalt-combining constituents which could compete with the cells for the free cobalt ions and thus effect a diminished uptake of cobalt by the cells, we employed a defined growth medium free of cobalt-combining substances. In a medium containing 0·1 % (w/v) glucose, 0·1 % (w/v) $(NH_4)_2SO_4$, 30 μg./ml. nicotinamide, 100 μg./ml. $MgSO_4.7H_2O$ in 0·001 M-K_2HPO_4 pH 7·3 the uptake of cobalt by the cells was again considerably less (one-third to one-quarter) than that shown by cells suspended in phosphate buffer alone. The removal of glucose and/or $(NH_4)_2SO_4$ from the complete medium did not significantly alter the amount of cobalt bound by the cells. Further, when an aged inoculum of viability less than 0·001 % was used, a similar difference was again found in the amount of cobalt bound by these cells in the complete defined medium and in 0·001 M-K_2HPO_4. From these results, it appeared doubtful whether the change in metabolic state associated with the ability of the cells to reproduce themselves would account for the wide differences found in cobalt uptake.

Antagonistic action of magnesium

We next investigated the effect of magnesium on the amount of cobalt taken up by the cells. When magnesium was added to the phosphate buffer in a concentration equivalent to that used in the defined medium, 10 μg. Mg^{++}/ml., a marked decrease in the amount of cobalt bound by the cells was observed. Increasing the concentration of Mg^{++} from 10 to 1000 μg./ml. resulted in a further diminished uptake of cobalt by the cells (Table 4). The extent of the antagonism was such that between the limits of 10 and 1000 μg. Mg^{++}/ml., a tenfold increase in the concentration of magnesium resulted in nearly a fourfold decrease in the amount of cobalt bound by the cells. Independent studies of the extent of growth inhibition effected by cobalt at increasing levels of magnesium in the defined medium showed that as the concentration of magnesium in the medium was raised, increased concentrations of cobalt were required to prevent growth of the cells.

Table 4. *Cobalt uptake as affected by concentration of magnesium in the medium*

0·618 mg. dry wt. cells inoculated into 10·0 ml. 0·001 M-K_2HPO_4, pH 7·4, containing 0·4 μc. radio-active cobalt in final concentration of 10 μg. CO^{++}/ml. and varying concentrations of Mg^{++} as $MgSO_4.7H_2O$. Time of incubation 1 hr. at 37°.

Concentration of Mg^{++} (μg./ml.)	Conc. of cells (mg. dry wt./ml.)	Co^{++} uptake (μg. Co^{++}/mg. dry wt. cells)
0	0·06	18·3
10	0·06	7·68
100	0·06	1·90
1000	0·06	0·48

Cobalt uptake under anaerobic conditions

The uptake of cobalt by cells of *P. vulgaris* under anaerobic conditions was studied by passing a continuous stream of oxygen-free nitrogen gas through the inoculated medium (1:5 MEB) contained in 50 ml. capacity plastic centrifuge tubes. Oxygen-free solutions of cobalt were added by means of a syringe. Control tubes were treated in an identical manner but with the nitrogen gas replaced by compressed air. At the end of the incubation period the tubes were capped immediately to maintain the gas phase unchanged during time of centrifugation.

Table 5. *Uptake of cobalt under anaerobic conditions*

20 ml. 1:5 MEB containing 1·0 μc. radio-active cobalt in final concentrations of 50 μg. Co^{++}/ml., inoculated with: (*a*) 2·76 mg. dry wt. of cells; (*b*) 4·2 mg. dry wt. cells. Incubation period 1 hr. at 37°.

Gas phase	Cell concentration (mg. dry wt./ml.)	Co^{++} uptake (μg. Co^{++}/mg. dry wt. cells)
(*a*) Air	0·0138	4·15
N_2	0·0138	2·34
(*b*) Air	0·21	6·6
N_2	0·21	4·7

The results of two such experiments are summarized in Table 5. In each case, sufficient cobalt (50 μg./ml.) was present in the medium to prevent growth of both aerobic and anaerobic cultures. Under these conditions, cells maintained in a nitrogen atmosphere bound less cobalt/unit of cellular material than did the cells maintained in the presence of air. These results are of interest since it has been previously found that the concentration of cobalt required for growth inhibition of anaerobic cultures of Proteus is 2 to 3 times that required to prevent growth of similar inocula cultured in air (Schade, 1949).

Cobalt uptake by a cobalt resistant strain of P. vulgaris

By repeated transfer of inocula of our cobalt sensitive strain of *P. vulgaris* into media (MEB) containing increasing concentrations of cobalt, we obtained a strain capable of growth in the presence of 150 μg. Co^{++}/ml. The amount of cobalt bound by these adapted cells was compared with that bound by the parent strain. Tests were run in 1:5 MEB at two levels of cobalt concentration, 10 and 200 μg./ml., corresponding to growth inhibitory levels for the stock and cobalt-resistant strain, respectively. The results of two experiments are given in Table 6.

Although the cobalt-sensitive strain fixed approximately 1·5 times more cobalt than did the resistant strain at both levels of cobalt concentration used, the resistant strain bound, at 200 μg. Co^{++}, thirty times as much cobalt as the sensitive strain bound at its growth inhibitory level of 10 μg. CO^{++}/ml. From these results, it seems doubtful whether the failure of 150 μg. CO^{++}/ml. to inhibit growth of the resistant strain can be ascribed merely to failure of the cells to bind significant quantities of the metal.

Table 6. *Cobalt uptake by* P. vulgaris, *comparison of stock strain with cobalt resistant variant*

10·0 ml. of 1:5 MEB containing $CoSO_4.7H_2O$ in concentration of 10 and 200 μg. Co^{++}/ml. and 0·49 μc. radio-active cobalt inoculated with approximately 1·0 mg. dry wt. cells of each strain. Time of incubation 1 hr. at 37°.

	Concentration of cobalt in the medium			
	10 μg./ml.		200 μg./ml.	
Cell type	Cell conc. (mg. dry wt./ml.)	Co^{++} uptake (μg.Co^{++}/mg. dry wt.)	Cell conc. (mg. dry wt./ml.)	Co^{++} uptake (μg. Co^{++}/mg. dry wt. cells)
Co R	0·073	0·977	0·071	43·6
Co S	0·07	1·432	0·076	76·7
Co R	0·121	0·775	0·105	39·1
Co S	0·087	1·361	0·095	59·9

Co R = cobalt resistant; Co S = cobalt sensitive

Cobalt uptake as affected by the time of exposure and the pH *of the medium*

Employing a concentration of 100 μg. Co^{++}/ml. of 1:5 MEB, we determined the amount of cobalt bound by cells suspended in this medium for 15, 30, 60 and 120 min. No significant increase in cobalt uptake by cells suspended for periods longer than 30 min. was observed; 70–80 % of the maximum amount taken up could be accounted for in the cells at the end of only 15 min. exposure.

The effect of the pH value of the medium on cobalt uptake by the cells was tested in 0·01 M potassium phosphate buffer. At concentrations of 5 μg. CO^{++} and of 0·07 mg. dry wt. cells/ml. medium, the amount of cobalt bound/unit of cellular material was 0·75 μg. at pH 5·0, 1·02 μg. at pH 6·0 and 1·65 μg. at pH 7·0. Efforts to test the effect of higher pH values on cobalt uptake in this medium were complicated by precipitation of insoluble cobalt compounds.

Removal of cobalt from the cells

How much of the cobalt bound to the cell substance was readily removable with water or HCl was investigated. A quantity of cells (1·43 mg. dry wt.) was suspended in 2·0 ml. distilled water containing 1 μc. radioactive cobalt in a final concentration of 10 μg. Co^{++}/ml. After incubation at 37° for 1 hr. the suspensions were centrifuged, supernatants removed, and the cells washed four times by repeated centrifugation and suspension in 10 ml. distilled water. The amount of cobalt taken up by the cells prior to the first wash was determined by summation of the counts found in the supernatant of each wash and the number remaining in the washed cells. Correction for cobalt retained in adhering supernatant was made. Of a total 3·91 μg. Co^{++} taken up by the cells, 46, 32, 28 and 26 % remained with the cells after the first, second, third and fourth washes, respectively. Hence, it is apparent that 20–25 % of the cobalt originally associated with the cells was bound more or less fixedly to the cellular substance.

When fresh cells exposed to similar concentrations of cobalt were subsequently suspended in 0·1 N-HCl, 95–98 % of the total cobalt taken up appeared in the supernatant.

DISCUSSION

The observation that cells of *P. vulgaris* take up cobalt rapidly and that distilled water or 0·1 N-HCl easily remove it indicates that cobalt is primarily bound with the cell substance in a readily reversible manner. Such binding may be accomplished by the mechanism of ion adsorption with the cells acting in a manner similar to charged colloidal particles, or by direct chemical combination with reactive groups throughout the cell as well as with those found at the cell surface.

Evidence for the existence of amino-acid groups at the surface of bacterial cells has been presented by Cohen (1945). Chelation of such groups with cobalt may represent one mode of attachment of the metal to the cell. Albert (1950) measured the affinity of a number of divalent amino-acids for heavy metal ions, including cobalt, and found a degree of stability for all amino-acids: $Cu^{++} > Ni^{++} > Zn^{++} > Co^{++} > Cd^{++} > Fe^{++} > Mn^{++} > Mg^{++}$. The ability of cobalt to form complexes with histidine at physiological pH values has been shown by Hearon, Burk & Schade (1949). The first reaction product formed, cobalt dihistidine, as well as the oxygenated product oxy*bis*cobaltodihistidine, are completely reversible with HCl, but in time, in the presence of oxygen, the latter product is converted to an irreversible complex. It is conceivable that this type of bond may in part account for the small portion of cobalt remaining in the cells of *P. vulgaris* after treatment with HCl. Further, cobalt may be combined irreversibly with cysteine (Michaelis & Barron, 1929).

Reversible uptake of cations from solution by bacteria has been investigated by a number of workers. McCalla (1940) studied the uptake of H^+, methylene blue and Mg^{++} by a number of soil bacteria and found, for example, that Mg^{++} taken up by *Bact. coli* could be removed to a significant degree by the subsequent addition of Co^{++}, Ba^{++}, or Mn^{++}. Valko & Dubois (1944) obtained a reversal of the bactericidal action of surface-active cations by detoxification with surface-active anions or by non-toxic surface-active cations. In these instances the uptake of cations was explained as the result of adsorptive forces exhibited by the negatively charged cells.

The ability of magnesium to alter the amount of cobalt taken up by the cells of *P. vulgaris* and to affect the concentration of cobalt required for growth inhibition may be an expression of competition of the two ions for an essential metabolic group on the cell. Abelson & Aldous (1950) reported a similar action of magnesium for nickel as well as cobalt with *Bact. coli* and found that Mg^{++} exerted its effect at the time of metal uptake. The inability of magnesium to replace cobalt already bound by the cell may be due to a difference in the stability of the metal complexes formed with the cell substance.

This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U.S. Public Health Service.

REFERENCES

ABELSON, P. H. & ALDOUS, E. (1950). Ion antagonisms: interference of normal magnesium metabolism by nickel, cobalt, cadmium, zinc and manganese. *J. Bact.* **60**, 401.

ALBERT, A. (1950). Quantitative studies of the avidity of naturally occurring substances for trace metals. L-Amino-acids having only two ionizing groups. *Biochem. J.* **47**, 531.

COHEN, S. S. (1945). The chemical alteration of a bacterial surface with special reference to the agglutination of *B. proteus* OX-19. *J. exp. Med.* **82**, 133.

HEARON, J., BURK, D. & SCHADE, A. L. (1949). Physical chemical studies of reversible and irreversible complexes of cobalt, histidine and molecular oxygen. *J. nat. Cancer Inst.* **9**, 337.

LEVY, H. B., SKUTCH, E. T. & SCHADE, A. L. (1949*a*). The effect of cobalt on the relationship between nucleic acid concentration and growth rate in *Proteus vulgaris*. *Arch. Biochem.* **24**, 199.

LEVY, H. B., SKUTCH, E. T. & SCHADE, A. L. (1949*b*). Effect of cobalt on the phosphorus turnover rate in the nucleic acids of *Proteus vulgaris*. *Arch. Biochem.* **24**, 206.

McCALLA, T. M. (1940). Cation adsorption by bacteria. *J. Bact.* **40**, 23.

MICHAELIS, L. & BARRON, E. S. G. (1929). Comparative study of the complexes of cysteine with the metals of the iron group. *J. biol. Chem.* **83**, 191.

SCHADE, A. L. (1949). Cobalt and bacterial growth with special reference to *Proteus vulgaris*. *J. Bact.* **58**, 811.

VALKO, E. I. & DUBOIS, A. S. (1944). The antibacterial action of surface active cations. *J. Bact.* **47**, 15.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Fourteenth General Meeting (*the Annual General Meeting*) *on Tuesday, Wednesday and Thursday, the* 15*th*, 16*th and* 17*th of April* 1952, *at Oxford University. The following communications were made:*

COMMUNICATIONS

SYMPOSIUM ON THE NATURE OF VIRUS MULTIPLICATION

Problems of Protein Synthesis. By H. Chantrenne (*Université Libre de Bruxelles*)

Virus Multiplication considered as a form of Protein Synthesis. By F. C. Bawden and N. W. Pirie (*Rothamsted Experimental Station, Harpenden*)

Metabolic Aspects of Virus Multiplication. By D. J. Bauer (*Wellcome Laboratories of Tropical Medicine, London*)

Nucleic Acids in Virus Multiplication. By Roy Markham (*Molteno Institute, University of Cambridge*)

An Analysis of Bacteriophage Multiplication. By S. E. Luria (*University of Illinois*)

The Nature of Phage Reproduction. By André Lwoff (*Institut Pasteur, Paris*)

The Role of Mutation in the Survival and Multiplication of Bacterial Viruses. By J. S. K. Boyd (*Wellcome Laboratories of Tropical Medicine, London*)

The Multiplication of Bacterial Viruses studied by Radiobiological Means. By Raymond Latarjet (*Institut du Radium, Paris*)

Divalent Metals in Phage Production. By Paul Fildes, D. Kay and W. K. Joklik (*University of Oxford*)

Aspects of the Formation of Incomplete Virus. By Sven Gard (*Karolinska Institutet, Stockholm*)

The Multiplication of the Influenza Virus considered in relation to the General Problem of Biological Multiplication. By L. Hoyle (*General Hospital, Northampton*)

Growth of Viruses of the Influenza Group as revealed by Electron Microscopy of Infected Cells. By T. H. Flewett (*University of Leeds*)

The Argument for a Venerable Hypothesis of Virus Multiplication. By Forrest Fulton (*London School of Hygiene and Tropical Medicine*)

The Multiplication of Insect Viruses. By G. H. Bergold (*Laboratory of Insect Pathology, Sault Ste. Marie, Ontario*)

The Multiplication of the Fowl Tumour Viruses. By J. G. Carr (*Poultry Research Centre, Edinburgh*)

Multiplication Cycles in Neurotropic Viruses. By F. K. Sanders (*University of Oxford*)

ORIGINAL PAPERS

The Use of the Neufeld Capsular Swelling Reaction in the Identification of Rumen Streptococci *in situ*. By Margaret Macpherson and A. E. Oxford (*Rowett Research Institute, Aberdeenshire*)

Environmental conditions in the sheep's rumen are complex and different from those of ordinary bacteriological media. It is therefore desirable to have direct microscopical confirmation of the presence in the rumen of any presumed isolate which has been obtained in pure culture. For this purpose we have used the Neufeld capsule swelling reaction, originally described for the *Pneumococcus* by Neufeld (1902) and later used by Mirick, Thomas, Curnen & Horsfall (1944) for the identification of an apparently non-mucoid *Streptococcus* M.G. closely related to *Strep. salivarius*. Since one of us (M.M.) had previously isolated a number of starch fermenting streptococci from the rumen of concentrate-fed sheep, our first experiments were carried out with pure cultures of these organisms. When the bacterial suspension and homologous antiserum were mixed together on a slide, the capsules immediately became large and optically defined. On the other hand, if normal serum or immune serum prepared against *Strep. bovis* (NCTC. 8177) was used no encapsulation was revealed even after long standing.

The reaction still took place if a pure culture of the rumen *Streptococcus* was first suspended in strained rumen liquor with its normal microflora, the morphology of the added *Streptococcus* showing a positive Neufeld reaction sufficiently distinct to enable it to be easily distinguished from other rumen bacteria including different streptococci, which were present in the same preparation.

The same antiserum has likewise been used to demonstrate the presence of encapsulated antigenically related streptococci in rumen contents of another sheep fed on a relatively starch-free hay diet.

REFERENCES

Mirick, G. S., Thomas, L., Curnen, E. C. & Horsfall, F. L. (1944). A non-haemolytic Streptococcus from the respiratory tract. *J. exp. Med.* **80**, 391.

Neufeld, F. (1902). Die Agglutination der Pneumokokken. *Z. Hyg. InfektKr.* **40**, 54.

Studies on the Macromolecular Organization of Bacterial Extracts. By R. Y. Stanier, H. K. Schachman and A. B. Pardee

Five different methods of preparing extracts from bacterial cells yielded similar distributions of macromolecular constituents as indicated by ultracentrifugal patterns. Extracts from a number of different bacteria showed essentially similar patterns. Three principal components were found, with sedimentation constants of approximately 40, 29, and 5 S. In many extracts, it was evident that the most slowly moving peak consisted of two components, a broad diffuse boundary and a sharp spike containing desoxypentose nucleic acid. Extracts from the photosynthetic bacterium *Rhodospirillum rubrum* contained, in addition to the above-mentioned components, a component of very large size (sedimentation constant approximately 190 S), with which the entire pigment complement of the cells was associated. Chemical analyses of crudely fractionated extracts showed that the bulk of the pentose nucleic acid of the cells was associated with the particles of sedimentation constants from 20 to 40 S, while the desoxypentose nucleic acid was almost all present in the fraction of lower sedimentation rate.

The Thermostability of Penicillinase. By Eileen E. D. Manson and M. R. Pollock (*National Institute for Medical Research, London*)

The stability at 100° of five commercial penicillinase preparations, which in all cases were cell-free culture filtrates, has been investigated. Three of these preparations retained about 80 % of their original activity after 2 min. and 20 % after 1 hr. The other two were completely inactivated after treatment for 2 min.

Cultures used for the production of the three stable preparations and one of the unstable preparations were obtained and their penicillinases tested under different conditions. All strains belonged to the *Bacillus* genus. The penicillinase preparations, produced by culture of these four strains in casein hydrolysate medium, were rapidly inactivated, less than 30 % of their original activity remaining after 2 min. The addition of 1 % (w/v) gelatin to these casein hydrolysate preparations gave marked protection to the penicillinase in every case, 70–80 % of activity remaining after 2 min. The addition of a concentrate of infusion broth also had the same protective effect as gelatin. Penicillinase prepared from culture in beef tryptic digest broth behaved similarly to the casein hydrolysate plus 1 % gelatin preparations.

Similar results were obtained when the stability at 100° of penicillinase from *B. cereus* NRRL 569 was tested, but the protection by gelatin and broth was reduced, only 40–50 % of the activity remaining after 2 min. This may be caused by gelatinase activity of the culture supernatants as this was the only strain of those tested which liquefied gelatin.

It is suggested that the heat stability of penicillinase depends on the constituents of the medium present during the heat treatment.

The Significance of Pleomorphism in *Mycobacterium tuberculosis*. By C. H. Lack (*Institute of Orthopaedics, London*)

Genetic Recombination in *Bacterium coli* K 12: Observations on the Nature of the Gamete and the Mode of its Transmission. By William Hayes (*Postgraduate Medical School of London*)

The Genetic Significance of certain L-forms of *Bacterium coli*. By W. Howard Hughes (*Wright-Fleming Institute of Microbiology, London*)

In a previous communication (1952) the work of Demerec (1948) was confirmed using single-cell cultures of *Staphylococcus aureus*. It was found that a wide range of variation in penicillin sensitivity was present in the descendants of single cells. It was difficult to understand how this could be due to mutation alone as the frequency appeared too great, yet it was clear that no new genetic material could have been introduced into the cultures.

Recently an observation has been made which helps to explain this. A strain of *Bact. coli*, Faecal Type 1 (Brent), was cultured by Fleming's method (1950) on slides and the development of colonies from the single cell followed. It was noticed that small a proportion, rather over 3 %, developed into L-forms, that about 80 % developed into normal colonies and that the remaining 16 % gave colonies consisting of both types of organism. In these latter colonies the first division of the organism appeared to be normal but in one of the two daughter cells division was suppressed while in the other it proceeded normally. That organism in which suppression of division occurred became an L-form while the other gave a series of normal cells.

This obvious unequal division has not been seen to take place except at this early stage in the development of the colony.

If from such a culture of mixed forms the L-forms are selected and grown as single cells in a normal environment, among their offspring, the proportion of L-forms is increased. The small cells give cultures with fewer L-forms than the original strain when seeded to microcultures. In no case yet has a small form failed to give any L-forms nor has an L-form failed to give a few normal colonies.

It is possible by this method to see a single organism divide into two daughter cells which react differently to an identical environment. No other differences have been noticed between the selected strains.

REFERENCES

Demerec, M. (1948). Origin of bacterial resistance to antibiotics. *J. Bact.* 56, 63.

Fleming, A., Voureka, A., Kramer, I. R. H. & Hughes, W. H. (1950). The morphology and motility of *P. vulgaris* and other organisms cultures in the presence of penicillin. *J. gen. Microbiol.* 4, 257.

Hughes, W. Howard (1952). Variation in penicillin resistance in single-cell cultures of *Staphylococcus aureus*. *J. gen. Microbiol.* 6, 175.

On the Staphylolytic Action of Actinomycetin. By J. M. Ghuysen and M. Welsch. (*Université de Liége et C.R.P.A.*)

Culture-filtrates from *Streptomyces albus* G (actinomycetin) dissolve heat-killed bacteria (*Escherichia coli*) and sometimes 'living' ones (*Staphylococcus aureus*). Activity against dead *E. coli* is often found alone, but activity against living *S. aureus* was never found in the absence of the former. However, the staphylolytic activity is not quantitatively related to the activity against dead *E. coli*.

A single agent (actinozyme) is believed to act on both substrates, but something more is needed in addition for staphylolysis.

Staphylolytic fluid can be obtained by freezing and thawing agar cultures or, more conveniently, by filtration or centrifugalization of cultures in liquid medium (salts from Czapek and 1 % peptone), incubated at 27°, on a shaker, for 3–4 days.

The amount of staphylococci dissolved in a given time (measured by nephelometry) is a linear function of the square root of the relative concentration of the staphylolytic principle (Schütz's rule). This furnishes a convenient method for titration.

The lytic agent can be concentrated by salt precipitation, followed by dialysis. Maximal activity requires an optimal ionic strength (around 0·013) and the presence of specific salts, notably phosphate. In the presence of a minimal amount of phosphate, the following salts, used singly, to complete the ionic strength to optimal level, give best results in the following order: $NaCl$, NH_4Cl, K_2SO_4, Na_2SO_4, $(NH_4)_2SO_4$.

Iron and the Nitrifying Bacteria. By JANE MEIKLEJOHN (*Rothamsted Experimental Station, Harpenden*)

Nitrosomonas europaea (Jensen's strain) and a strain of *Nitrobacter winogradskii*, isolated from a forest nursery soil, have been found to have very small absolute requirements for iron; both species will grow in the appropriate media purified with 8-hydroxyquinoline, and with no iron added. The addition of small amounts of iron to the purified media hastens the oxidation of ammonia or nitrite; the minimum dose giving this stimulating effect is 0·1 mg./l. for *Nitrosomonas* and 0·3 mg./l. for *Nitrobacter*. The stimulation of oxidation increases with increasing amounts of iron up to 6 mg./l. Both species can tolerate relatively large quantities of iron; in presence of M/500 ferrous sulphate (112 mg. Fe/l.) oxidation of ammonia or nitrite takes place normally. M/100 ferrous sulphate delays oxidation, but does not stop it.

Observations on Different Streptomycin Resistant Variants of *Bacterium coli*. By D. A. MITCHISON (*Postgraduate Medical School of London*)

The Formation of α-Ketoglutarate from Succinate by a Strain of *Pseudomonas fluorescens*. By MARGOT KOGUT (*The University of Sheffield*)

Washed suspensions of a strain of *Pseudomonas fluorescens*, KB 1, prepared from a culture grown at 25° on a liquid mineral medium with succinate as energy source, oxidize succinate rapidly (Q_{O_2}500–600). Using limited amounts of succinate (3–10 μmoles) the rate of oxygen consumption is linear until *c.* 1·5 moles O_2 per mole succinate have been used; thereafter the rate declines rapidly. Experiments with excess succinate in which both succinate disappearance and oxygen consumption were measured confirmed the ratio O_2/succinate of 1·5.

A search for possible end products showed the presence of small amounts of acetate and relatively large amounts of α-ketoacids. Paper chromatography of the 2:4-dinitro-phenylhydrazones with *n*-butanol-water as the solvent showed the presence of two compounds with R.F.'s similar to the hydrazones of pyruvate and α-ketoglutarate respectively. Oxaloacetate was excluded by heating to 100° for 10 min. in the presence of N-HCl prior to making the hydrazones.

Estimations of the α-ketoglutarate by the specific transaminase method of Krebs (1950) showed that during the oxidation of succinate α-ketoglutarate first accumulates and then disappears. The maximum yield expressed as moles of α-ketoglutarate produced per moles O_2 consumed was 0·154. The maximum theoretical yield on the assumption that succinate is quantitatively converted to α-ketoglutarate via citrate, would be 0·33.

Lockwood & Stodola (1946) report the production of α-ketoglutarate by a strain of *Ps. fluorescens* during growth upon glucose containing medium. When KB 1 is grown upon a succinate medium with a limited nitrogen supply, α-ketoglutarate, isolated as the 2:4-dinitrophenylhydrazone, accumulates.

These results seem to indicate the presence of a citric acid cycle mechanism in this organism, but with a partial block at the α-ketoglutarate oxidation step.

REFERENCES

KREBS, H. A. (1950). Manometric determination of L-aspartic acid in L-asparagine. *Biochem. J.* **47**, 605.

LOCKWOOD, L. B. & STODOLA, F. H. (1946). Preliminary studies on the production of α-ketoglutaric acid by *Pseudomonas fluorescens*. *J. biol. Chem.* **164**, 81.

Some Aspects of the Electron Microscopy of Leptospira.

BY J. W. CZEKALOWSKI and G. EAVES (*University of Leeds*)

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